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EXPRESSION AND FUNCTIONAL CHARACTERISATION OF THE REV GENE PRODUCT OF MAEDI VISNA VIRUS EV-1

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ABSTRACT OF THESIS

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The ovine Maedi Visna Virus (MVV) and the Human Immunodeficiency Virus Type 1 (HIV-1) are members of the *lentivirinae* retrovirus subfamily. Lentiviruses possess a complex genomic organisation, encoding several genes with a regulatory or auxiliary function. Alternative splicing of the genomic-length primary transcript is used to express this complexity. mRNA expression is subject to temporal regulation in both MVV and HIV-1. In HIV-1 a highly basic protein, Rev, mediates this regulation. Rev function requires binding to a highly structured RNA target, the Rev responsive element (RRE), and may involve facilitation of RNA nucleocytoplasmic export. Rev/RRE interaction is essential for virus replication. There is little overall sequence homology between lentivirus Rev proteins. However, functionally important basic and leucine-rich motifs are conserved. These domains are found within the putative MVV Rev protein, and a predicted RRE-like structure is present in a similar genomic location to that in HIV-1.

To compare the mode of action of MVV Rev with that of HIV-1 Rev, a series of functional assays were planned. The *rev* gene of a British isolate of MVV (EV-1) was cloned and sequenced. Recombinant Rev was expressed as a fusion protein in yeast and bacterial systems. A polyclonal antiserum directed against a synthetic Rev polypeptide was generated to aid purification. Expression in yeast was characterised by a low product yield, due to the highly toxic nature of the Rev fusion protein. Alternative expression and purification protocols were unable to greatly improve the yield and purity of product. The bacterial pGEX system, in which Rev was fused to glutathione S-transferase (GSTRev), was employed as an alternative. Purification by affinity chromatography resulted in an improved yield and purity of product. Partial instability of the fusion protein may have resulted in observed contamination.

Binding of GSTRev to RNA corresponding to the predicted RRE was assayed by filter binding experiments. A specific vector context and low temperature were required to generate high quality RNA. GSTRev bound with high affinity to RRE-RNA, but not to RNA corresponding to antisense RRE. Addition of a non-specific competitor RNA reduced binding to antisense, but not sense, RNA.

Rev is the least well conserved protein amongst sequenced isolates of MVV. To test for the functional conservation of the Rev/RRE axis, the cross reactivity of Rev function on heterologous RREs was examined by transient transfection assay. Whilst cross-strain functional reciprocity was observed, both the EV-1 and 1514 isolate Rev proteins demonstrated greatest activity on cognate RRE. Co-divergence of the *rev* gene and RRE structure of each strain has therefore occurred. MVV Rev was able to function through the RRE of the closely related caprine arthritis encephalitis virus. These results may have implications for the possible development of anti-lentiviral gene therapy based on *trans*-dominant, inhibitory Rev molecules.

In memory of my grandfather, William Peter Copland (1918-1991)

DECLARATION

I declare that the composition of this thesis and the experiments described are my own work, unless otherwise stated with due acknowledgement. No part of this work has been, or will be, submitted for any other degree, diploma or qualification.

Michael William Fotheringham
November 1995

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LIST OF ABBREVIATIONS

AD	activation domain
AIDS	acquired immunodeficiency syndrome
amp	ampicillin
APS	ammonium persulphate
β-Gal	β-galactosidase
BD	basic domain
BIV	bovine immunodeficiency virus
BLV	bovine leukaemia virus
bp	base pairs
BSA	bovine serum albumin
CAEV	caprine arthritis-encephalitis virus
CAT	chloramphenicol acetyltransferase
CIP	calf intestinal phosphatase
CNS	central nervous system
cpm	counts per minute
CRS	*cis*-acting repressive sequence
Δ	deletion
DEPC	diethyl pyrocarbonate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
E. coli	*Eschericia coli*
EDTA	ethylenediamine tetra-acetatic acid
EIAV	equine infectious anaemia virus
EM	electron microscopy
FCS	foetal calf serum
FIV	feline immunodeficiency virus
g	gravity
gal	galactose
GST	glutathione S transferase
HBV	hepatitis B virus
HIV	human immunodeficiency virus
hnRNP	heterogeneous nuclear ribonucleoprotein
HTLV	human T-cell leukaemia virus
IPTG	β-D-isopropyl thiogalactopyranoside
kb	kilobase
kd	dissociation constant
kDa	kilo Dalton

LB	Luria broth
LTR	long terminal repeat
mRNA	messenger RNA
MVV	maedi visna virus
MW	molecular weight
NLS	nuclear localisation signal
NOS	nucleolar localisation signal
nt	nucleotide(s)
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PMSF	phenylmethyl sulphonyl fluoride
RBE	rev binding element
RNA	ribonucleic acid
RNase	ribonuclease
RNP	ribonucleoprotein
rNTP	ribonucleotide triphosphate
RRE	rev responsive element
RSV	Rous sarcoma virus
RxRE	rex responsive element
SA-OMVV	South African ovine maedi visna virus
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulphate
SIV	simian immunodeficiency virus
SL	stem loop
SPBS	sterile phosphate buffered saline
TAE	tris-acetate/EDTA
TAR	*trans*-activating region
TBE	tris-borate/EDTA
TD	*trans*-dominant
TEMED	tetramethylethylene diamine
Ty	retrotransposon yeast
ura	uracil
UTP	uridine triphosphate
UV	ultra violet
VLP	virus-like particle
v/v	volume:volume ratio
w/v	weight:volume ratio

CHAPTER ONE

INTRODUCTION

1.1 The Lentiviruses

The *lentivirinae* subfamily of exogenous, non-oncogenic retroviruses establish life-long infections in host organisms. These viruses are so named due to the extended asymptomatic period, insidious onset and relentless nature of disease (*lentus*: slow Latin). Lentiviruses share the ability to persist within the host during the asymptomatic period despite vigorous immune responses. Cells of the immune system are targets for infection, and resulting destruction and/or dysregulation of the immune system is responsible for pathology. Retroviruses possess an RNA genome, and replicate via a DNA intermediate, which characteristically integrates into the host genome to form the provirus.

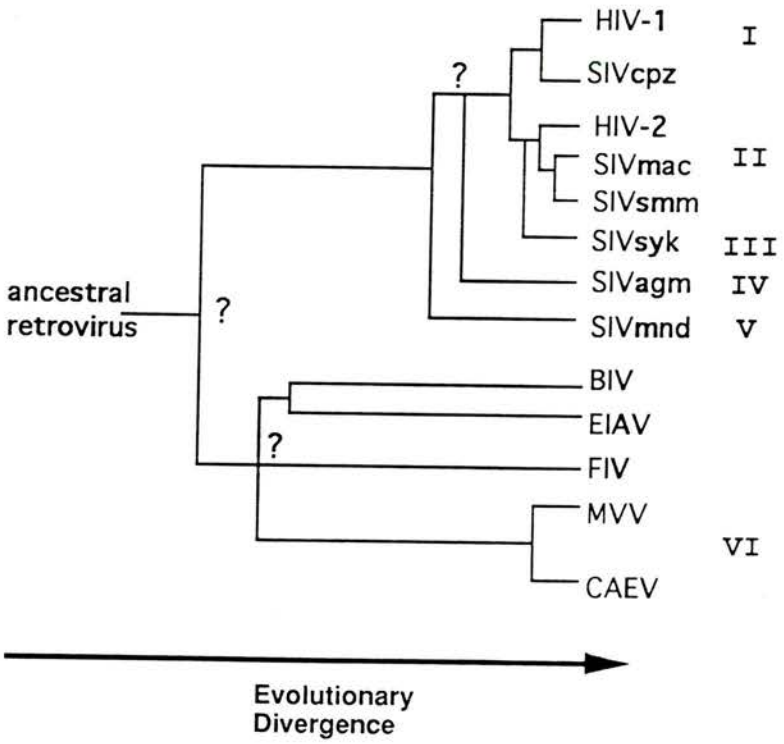
The prototypic member of the lentivirus genus is the ovine Maedi-Visna virus (MVV). Importation of sheep inapparently infected with MVV was responsible for epidemics of progressive interstitial pneumonia (*maedi* in Icelandic) and progressive paralysis (*visna* in Icelandic) which devastated Icelandic sheep flocks between 1939 and 1952 (Sigurdsson, 1954). The single viral aetiology of these diseases was demonstrated in 1967 (Gudnadottir and Palsson, 1967). MVV is also known as ovine progressive pneumonia virus (OPPV) (Cutlip and Laird, 1976) or ovine lentivirus (OvLV) (Lairmore *et al.*, 1986). Other members of the lentivirus genus include equine infectious anaemia virus (EIAV) (Dreguss and Lombard, 1954), bovine immunodeficiency virus (BIV) (Van der Maaten *et al.*, 1972), caprine arthritis encephalitis virus (CAEV) (Crawford *et al.*, 1980), simian immunodeficiency virus (SIV) (Daniel *et al.*, 1985), feline immunodeficiency virus (FIV) (Pederson *et al.*, 1987), and human immunodeficiency virus (HIV) type 1 (Barre-Sinoussi *et al.*, 1983) and type 2 (Clavel *et al.*, 1986). The field of lentivirus research was stimulated by the lentiviral classification of HIV-1, the primary aetiological agent of the current human pandemic of acquired immunodeficiency syndrome (AIDS) (Chiu *et al.*, 1985). Subsequent research effort has focused on HIV. Throughout this thesis the literature concerning HIV will be extensively quoted.

1.2 Lentiviral Phylogeny

Traditional approaches to the decipherment of the evolutionary relationships of the lentiviruses have been based on phylogenetic tree analysis using sequence data obtained from conserved sections of the genome, for example the reverse transcriptase region of *pol* (see

figure 1.1). This is hindered by the extremely high evolutionary rates achieved by lentiviruses (~10^6 fold faster than the DNA genomes of 'higher' organisms) (Gojobori *et al.*, 1990). A consensus has emerged that the primate lentiviruses form five groups within a distinct clade (see legend to figure 1.1) (Huet *et al.*, 1990; Myers *et al.*, 1992; Hirsch *et al.*, 1993), and that the ovine and caprine lentiviruses form a second clade (Querat *et al.*, 1990). The relatively recent time scale for lentiviral radiation is supported by most authors. Querat and colleagues suggest that the divergence of the ungulate and primate viruses occurred ~430 years ago (1990). However, the position of the non-primate lentiviruses remains uncertain.

Figure 1.1: Evolutionary Relationship of the Lentiviruses



The evolutionary relationships of the lentivirus family. An adaptation from published evolutionary tree analyses, based on data from *gag* coding sequences (Garvey *et al.*, 1990; Querat *et al.*, 1990; Myers *et al.*, 1992; Hirsch *et al.*, 1993). Analyses concur on the relationships of the primate viruses; the position of the non-primate viruses is less clear. Question marks (?) identify divergence points of greatest uncertainty. Branch length is for clarity only, and is not to scale. SIV sequences isolated from African green monkeys (agm), mandrills (mnd), chimpanzee (cpz), rhesus macaques (mac), African Sykes' monkey (syk) and sooty mangabeys (smm). Consensus clades are marked with Roman numerals (I-VI). These groups, and prototype viruses are: **I** - HIV-1, **II** - HIV-2, **III** - SIV_{syk}, **IV** - SIV_{agm}, **V** - SIV_{mnd} and **VI** - MVV.

1.3 Pathology of MVV Infection

MVV infection is characterised by a prolonged prepatent period of between one to three years, although longer periods are possible (Sigurdsson, 1954). Subsequent clinical disease is generally progressive in nature, with any degree of regression of symptoms accompanied by inevitable recrudescence. Pathological lesions are typically of inflammatory origin, and may occur in a number of organs. Death normally occurs within 6-12 months of the onset of symptoms.

Animals suffering MVV disease typically present with loss of condition and respiratory distress (maedi). This is due to a lymphoproliferative interstitial pneumonia. Heavy accumulation of inflammatory cells within the pulmonary septa reduces the efficiency of gaseous exchange. The lung may swell to four times its normal mass (Sigurdsson, 1954). Severe lung disease may result in smooth muscle hyperplasia and the formation of lymphoid follicles with active germinal centres (Lairmore *et al.*, 1986).

Neurological disease (visna) occurs as a complication of maedi. It is characterised by locomotive dysfunction, leading to hind limb paralysis and occasionally paraplegia (Sigurdsson *et al.*, 1957). Inflammatory lesions within the central nervous system (CNS) lead to demyelination and gliosis (Sigurdsson *et al.*, 1957; Stowring *et al.*, 1985). Although lesions may extend into the grey matter, destruction of neurones is rare (Sigurdsson and Palsson, 1958). Meningitis may also occur (Oliver *et al.*, 1981).

Pathology in the mammary gland is similar to that in the lung, with chronic infiltration of inflammatory cells in the parenchyma and formation of germinal centres leading to chronic mastitis (Van der Molen *et al.*, 1985).

Chronic arthritis may occur as a further complication of maedi. Pathology in the joint involves swelling and calcification of soft tissue, perivascular lymphocytic infiltration and a degree of fibrosis and ossification (Oliver *et al.*, 1981). Arthritis is the dominant disease manifestation in adult goats of infection with CAEV (Crawford *et al.*, 1980). The carpal joint is typically affected, and in severe cases joint deformation may render limbs useless. CAEV disease also has pulmonary, neurological and mammary gland forms, although, unlike visna, caprine encephalitis is generally manifest in young animals and has a rapid course (Cork *et al.*, 1974).

It is unclear how viral replication relates to pathogenesis. It is clear that in the MVV infected sheep, pathological lesions are closely associated with sites of active virus expression

(Brodie *et al.*, 1995). The severity of CNS lesions can be reduced by host immunosuppression before infection (Nathanson *et al.*, 1976). Viral antigens are likely to drive the immune response, leading to inflammatory infiltration. Pathology might be the result of chronic stimulation of the immune system.

1.4 MVV Genomic Organisation

Information as to the genetic structure of MVV has been obtained by the sequencing of viral isolates. To date, six isolates of MVV have been sequenced. These are four variants of the Icelandic neurovirulent strain KV1514 (Sonigo *et al.*, 1985; Braun *et al.*, 1987; Staskus *et al.*, 1991b; Andresson *et al.*, 1993), the British isolate EV-1 (Sargan *et al.*, 1991) and the South African SA-OMVV (Querat *et al.*, 1990). These viruses demonstrate similar structures, although there is a high degree of sequence divergence. The EV-1 isolate has a 9203 base genome (Sargan *et al.*, 1991), and is representative of all sequenced isolates. Figure 1.2 illustrates the genomic structure of MVV: for comparison the structure of HIV-1 and the distantly related oncovirus, Human T-Cell Leukaemia Virus Type 1 (HTLV-1) (Poeisz *et al.*, 1980) are also shown. The basic features of retrovirus genomes are present. These include the flanking long terminal repeats (LTR), composed of the U3, R and U5 regions. U5 contains the complementary site for binding of the cellular tRNA ($tRNA_{1,2}^{lys}$) and U3 a polypurine tract, both of which are required for completion of viral replication (Sonigo *et al.*, 1985). Also contained within the LTR are sequences which direct and regulate the transcription of the viral genome.

MVV encodes six open reading frames (ORF) for the expression of viral proteins. Three of these, *gag*, *pol* and *env*, are homologous to genes common to all retroviruses. The remaining ORFs encode proteins with a putative regulatory or auxiliary function. This genomic complexity is a hallmark of the lentivirus genus. The additional ORFs are located between *pol* and *env*, and in the 3' *env* /LTR region. In MVV, these additional ORFs give rise to the proteins Tat, Rev and Vif.There is an additional ORF(W) in SA-OMVV. There is no evidence, however, for translation of this ORF (Querat *et al.*, 1990).

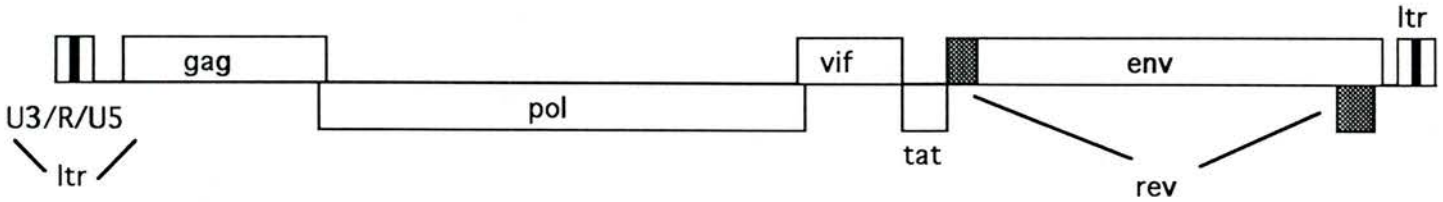
1.5 Viral Proteins

MVV expresses the basic complement of structural and enzymatic proteins common to

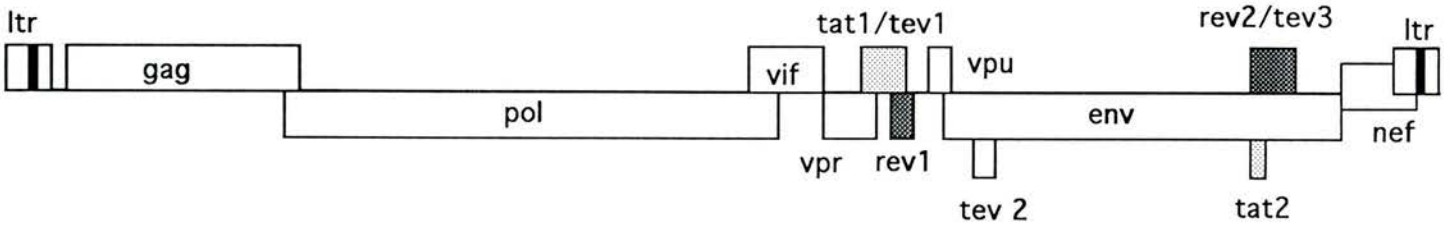
Figure 1.2: Genomic Organisation of Complex Retroviruses

The genomic structures of the lentiviruses, Maedi Visna virus (MVV) and Human Immunodeficiency virus type 1 (HIV-1), and of the oncovirus Human T-Cell Leukaemia virus type 1 (HTLV-1) are shown. Structures are to scale. Genomes are delineated by the long terminal repeats (LTR). This region contains three subdomains, U5, R and U3. Genes encoding structural (*gag*, *env*) or enzymatic (*pol*) proteins are shown. The *pro* (protease) coding region of the lentiviruses is contained within the *pol* gene, but is out of frame with *pol* in HTLV-1. Regulatory/auxiliary genes are present between the *pol*/*env* genes, and at the 3' end of the genomes. Bipartite genes are marked (tat/tax: light stippling, rev/rex: dark stippling). The tripartite gene *tev* in HIV-1 contains the *tat* first exon and *rev* second exon, and a unique coding exon within *env*.

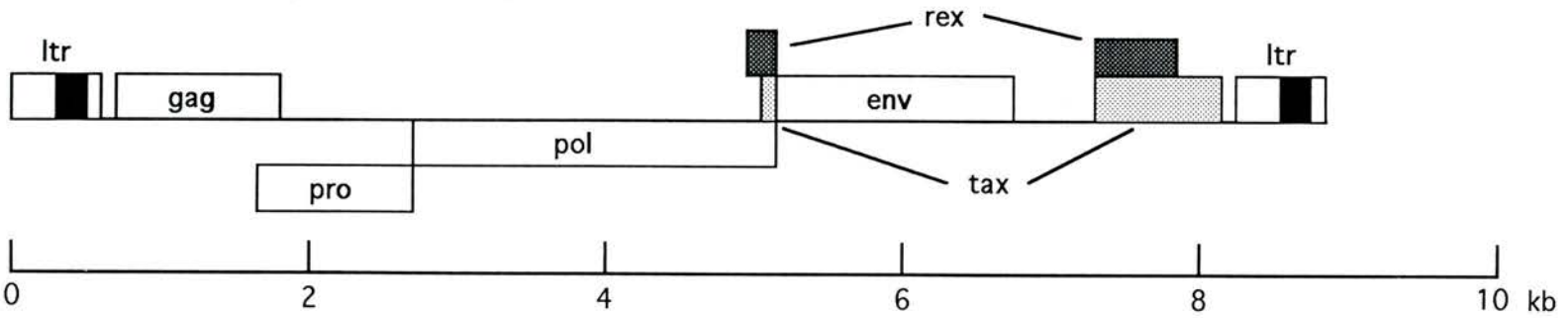
MAEDI VISNA VIRUS



HUMAN IMMUNODEFICIENCY VIRUS TYPE 1



HUMAN T-CELL LEUKAEMIA VIRUS TYPE 1



all retroviruses. These proteins are encoded by the *gag*, *pol* and *env* genes (Baltimore, 1974). Much of the data concerning MVV proteins is derived from the work of Vigne and colleagues (1982). However, the full range of viral activities remains to be completely characterised, and in several instances these have been inferred from comparison of the MVV coding capacity with that of other retroviruses.

The general strategy of retroviral protein expression is the production of polyprotein precursors from which functional activity is released by proteolysis. The major precursor of the MVV gag polypeptides is a non-glycosylated protein of 55kDa ($Pr55^{gag}$) (Vigne *et al.*, 1982). This is processed to generate the virion internal structural proteins. The matrix protein (MA, p16) interacts with the envelope. The HIV-1 equivalent, p17, directs the nuclear import of the viral pre-integration complex during the infection process (Bukrinsky *et al.*, 1993). The particle core is formed by multimerisation of the capsid protein (CA, p25). The highly basic nucleocapsid protein (NC, p14) binds to the viral genomic RNA within the core. MVV has no equivalent of the carboxy-terminal proximal Gag protein, p6, found in primate lentiviruses.

Gag sequences are also contained within a second 150kDa molecule (Vigne *et al.*, 1982). By analogy with other retroviruses, this is believed to be a Gag/Pol polyprotein precursor ($Pr150^{gag\text{-}pol}$), which is generated by a ribosomal frame shift event. A polypurine tract and RNA hairpin located within the *gag/pol* gene overlap mediates shifting of the ribosomal complex into the -1 reading frame. The ratio of Gag to Pol products is set by the frequency of this event. Pol derived polypeptides have enzymatic activities, including reverse transcriptase, protease and integrase. The MVV reverse transcriptase is Mg^{2+} dependent (Lin and Thormar, 1970) a property shared by all lentiviral enzymes, which distinguishes them from the Mn^{2+} dependence of type-C oncoviruses. MVV also contains a putative deoxyuridine triphosphatase (dUTPase) activity, a feature shared with CAEV, FIV and EIAV (McClure *et al.*, 1987; Elder *et al.*, 1992), but not by the primate or bovine lentiviruses. Loss of functional dUTPase activity is associated with severe restriction of viral replication in primary cells though not established cell lines in both FIV (Wagaman *et al.*, 1993) and EIAV (Lichtenstein *et al.*, 1995). dUTPase may act to both reduce the rate of misincorporation of dUTP for dTTP during DNA synthesis, and to increase the pool of dTTP in non-dividing cells where the concentration of dNTPs is low. MVV integrase activity has been characterised (Katzman and Sudol, 1994); however, production of the predicted protease has yet to be demonstrated.

A glycosylated 150kDa protein is the precursor of the MVV envelope proteins ($Pr150^{env}$) (Vigne *et al.*, 1982). There are 28 potential N-linked glycosylation sites within the Env protein (Sonigo *et al.*, 1985), and three hydrophobic regions characteristic of retroviruses (Kiyokawa *et al.*, 1984). The first of these acts as a signal peptide to target $Pr150^{env}$ to the cell membrane. It is not known whether this peptide is subsequently removed from the mature protein. A protease cleavage site divides Env into two components, the surface glycoprotein (SU) gp135, and the transmembrane protein (TM) gp46 (Vigne *et al.*, 1982). Gp135 is the putative ligand for the MVV cellular receptor, whereas gp46 is thought to mediate fusion.

Products of the small ORFs have been identified in MVV infected cells. These are Tat (10-11kDa) (Davis and Clements, 1989), Rev (19kDa) (Mazarin *et al.*, 1990) and Vif (29kDa) (Audoly *et al.*, 1992). These proteins will be discussed in detail later.

1.6 The Viral Lifecycle

Infection of a potential host cell is initiated by binding of the virion to a cellular receptor. Two putative receptor molecules for MVV have been described; an unidentified 50kDa protein (Crane *et al.*, 1991) and ovine MHC class II (Dalziel *et al.*, 1991). This antigen may form one component of the cellular receptor, since expression of class II is insufficient to mediate MVV susceptibility. The cellular receptor must be widely distributed: MVV infection has been reported in various cell types, including cells within the choroid plexus (Brahic *et al.*, 1981), oligodendrocytes and astrocytes in the CNS (Stowring *et al.*, 1985) and bronchiolar epithelial (Geballe *et al.*, 1985) cells. The major target for MVV *in vivo*, however, are cells of the monocyte/macrophage lineage (Narayan *et al.*, 1982; Gendelman *et al.*, 1985). MVV does not replicate within T lymphocytes (Gorrell *et al.*, 1992), a property shared by CAEV and EIAV, and one which differentiates these viruses from the primate, feline and bovine immunodeficiency viruses, for which T lymphocytes are a major host cell (Narayan and Clements, 1989). The cellular receptor for HIV-1 infection, CD4, has been characterised (Dalgleish *et al.*, 1984).

Binding to the cellular receptor is followed by fusion of the virion with the cell membrane, entry and uncoating. Reverse transcription of the genomic RNA then proceeds, primed by the binding of $tRNA^{lys}_{1,2}$ to the primer binding site in the LTR (Coffin, 1990); in HIV-1 this may occur within a virion-derived particle, demonstrating the importance of uncoating (Zhang *et al.*, 1995).

The double stranded DNA product of reverse transcription is the substrate for integration into the host genome. However, the role of integration in MVV infection is uncertain. A failure to detect integrated provirus in *in vitro* infected cells has been reported (Harris *et al.*, 1984). Vigne and co-workers did, however, observe integration during infection of foetal cornea cells (1985). Moreover, integrase deficient mutants of CAEV and HIV-1 are not infectious (Turelli *et al.*, 1994; Wiskerchen and Muesing, 1995). This supports the supposition that integration is an obligate step in the retroviral life cycle; re-examination of the MVV integration state using more sophisticated PCR analysis may resolve the issue.

1.6.1 *In Vitro* Replication

The integrated provirus is transcribed by the cellular RNA polymerase II. From this stage, the replicative cycles *in vivo* and *in vitro* diverge. *In vitro* infection of permissive cells, such as choroid plexus fibroblasts results in massive RNA amplification (up to 4000 copies per cell) (Brahic *et al.*, 1981) and extensive cytopathic effect (syncytia formation) induced by the production of viral Env protein. Virion formation requires packaging of two copies of the viral genomic RNA and the Gag and Pol proteins to form a viral capsid, which is surrounded by Env containing plasma membrane. The retrovirion is functionally diploid. The RNA packaging signal has not been described for MVV, however in other retroviruses, including HIV-1, an area of extensive predicted secondary structure in the 5' untranslated region (Ψ) has been shown to bind specifically to the Gag nucleocapsid polypeptide (Lever *et al.*, 1989; Darlix *et al.*, 1990; Harrison and Lever, 1992). Mature extracellular MVV virions are pleiomorphic, with diameters in the range 90-130nm, and possess condensed nucleoid cores (Thormar, 1961; Coward *et al.*, 1970).

1.6.2 The *In Vivo* Life Cycle: Restricted Replication

The lytic replicative cycle is not demonstrated by virally infected cells isolated from the host animal. Co-cultivation with permissive cells is required to recover virus from *in vivo* derived cells. Thus viral replication appears to be subject to an *in vivo* block: this phenomenon has been termed 'restricted replication' (Haase *et al.*, 1977; Brahic *et al.*, 1981). It has been hypothesised that restricted expression is the mechanism which allows lentiviral persistence in the host despite a vigorous specific immune response, and also explains the prolonged prepatent period (Haase, 1986).

The viral structural proteins appear to form the major targets for both the humoral and cell mediated immune responses against both MVV and HIV infection (Schrier *et al.*, 1989; Blacklaws *et al.*, 1994); however there is some evidence to suggest the presence of cytotoxic T cells directed against the regulatory protein Nef (Culmann *et al.*, 1989). Infected cells not expressing structural proteins might thus avoid immune system surveillance. This could be achieved by suppressed transcription or by a post-transcriptional mechanism, possibly based on the maintenance of subthreshold levels of the viral regulatory protein, Rev (see 1.11). It is thus possible to distinguish restricted replication (the presence of viral RNA and possibly regulatory proteins but no structural proteins) from true latency (little or no viral RNA) at the single cell level.

Results from early studies of animals infected with high experimental doses of MVV are consistent with the restriction hypothesis. Thus, intracranial injection with MVV was found to result in extensive (18%) infection of choroid plexus cells with viral DNA: however, structural proteins were detected in only a small fraction (0.1%) of these cells (Haase *et al.*, 1977). In a similar experiment, Brahic and colleagues observed viral infection of 1-3% of choroid plexus cells (1981). Viral RNA was detected in a similar proportion of cells, at copy numbers 100-fold lower than observed in lytic infection. Thus it was suggested that restricted transcription was at least partially responsible for the replicative block *in vivo*.

Infected monocytes derived from the blood-stream and CNS of naturally infected animals also display restricted replication. Differentiation of monocytes into tissue macrophages induces the transition from restricted to productive replication phases in both MVV and CAEV infection (Narayan *et al.*, 1983; Gendelman *et al.*, 1986). This mobile population of cells may thus act as the primary disseminating agents of MVV infection throughout the host organism: the 'Trojan Horse' hypothesis (Peluso *et al.*, 1985). Monocyte precursor cells within the bone marrow containing latently integrated provirus may form a reservoir for infection: infected monoblasts with very few viral RNA copies have been reported (Gendelman *et al.*, 1985).

The use of PCR based *in situ* localisation techniques have allowed the observation of previously undetectable single genomic copies of viral DNA. A reservoir of infection has been detected within the lymphoid organs of HIV infected individuals (Embretson *et al.*, 1993; Pantaleo *et al.*, 1993). It is clear that the majority of infected cells in lymphoid tissue (99-99.75%) are in a latent state, expressing no viral RNA (Embretson *et al.*, 1993). Using a pulmonary model of MVV infection, Haase and co-workers detected viral DNA in the majority of bronchiolar epithelial cells; however, only a fraction of these (10%) contained viral RNA, and this was present

at low copy number (Staskus *et al.*, 1991a). However, in both HIV and MVV infected individuals, there appears to be a small population of cells undergoing productive infection at all stages throughout infection: there is therefore no true clinical latency (Staskus *et al.*, 1991a; Pantaleo *et al.*, 1993). That these cells might be more numerous in the MVV infected host may be a consequence of higher basal level transcription in this virus (see section 1.10.tat). Seeding of the periphery with latently infected cells results in productive infection on cell differentiation (monocytes) or activation (lymphocytes). In turn, this may result in immune-mediated disease. In MVV infection this may take the form of dysregulation of the immune response, with immune cell infiltration and inappropriate release of mediating factors such as cytokines.

1.7 Viral Transcription

The MVV proviral LTR contains both promoter and enhancer activity (Hess *et al.*, 1985). Constructs containing a reporter gene under the control of the LTR are expressed in activated macrophages in transfected animals (Small *et al.*, 1989; Clements *et al.*, 1994). Thus it is likely that the regulation of MVV expression due to the activation state of the host cell is a function of sequences within the LTR. Putative elements have been mapped to the enhancer region within the U3 section of the LTR, and consist of potential binding sites for the transcription factors AP-1 and AP-4 (Hess *et al.*, 1989). The 1514 isolate of MVV contains six degenerate copies of the AP-1 site, four of which are contained within two 43bp repeat sequences. Only the TATA box proximal AP-1 site matches the consensus sequence. This site binds the AP-1 factor (a dimer of the factors Fos and Jun) (Gabuzda *et al.*, 1989; Shih *et al.*, 1992). Induction of cell activation, either by stimulation with serum or phorbol ester or during differentiation of monocytes into macrophages, is associated with rapid upregulation of the levels of these factors (Shih *et al.*, 1992). This supports the hypothesis that the *in vivo* restriction of viral expression is predominantly a transcriptional phenomenon. Significant variation in the sequence of the enhancer elements has been noted; in the British isolate EV-1 there is only one 43bp repeat and the TATA box proximal AP-1 site does not conform to consensus (Sargan *et al.*, 1995). Moreover, studies have found a lack of AP-1 binding activity to the EV-1 LTR, and implicated a novel ~40kDa transcriptional factor in the regulation of EV-1 LTR activity (Sutton *et al.*, submitted).

Transcription in activated cells or during lytic infection *in vitro* produces a genomic length, 9.4kb transcript. Differential splicing of the primary transcript results in the generation of a

Figure 1.3: Transcript Pattern of Maedi Visna Virus

A

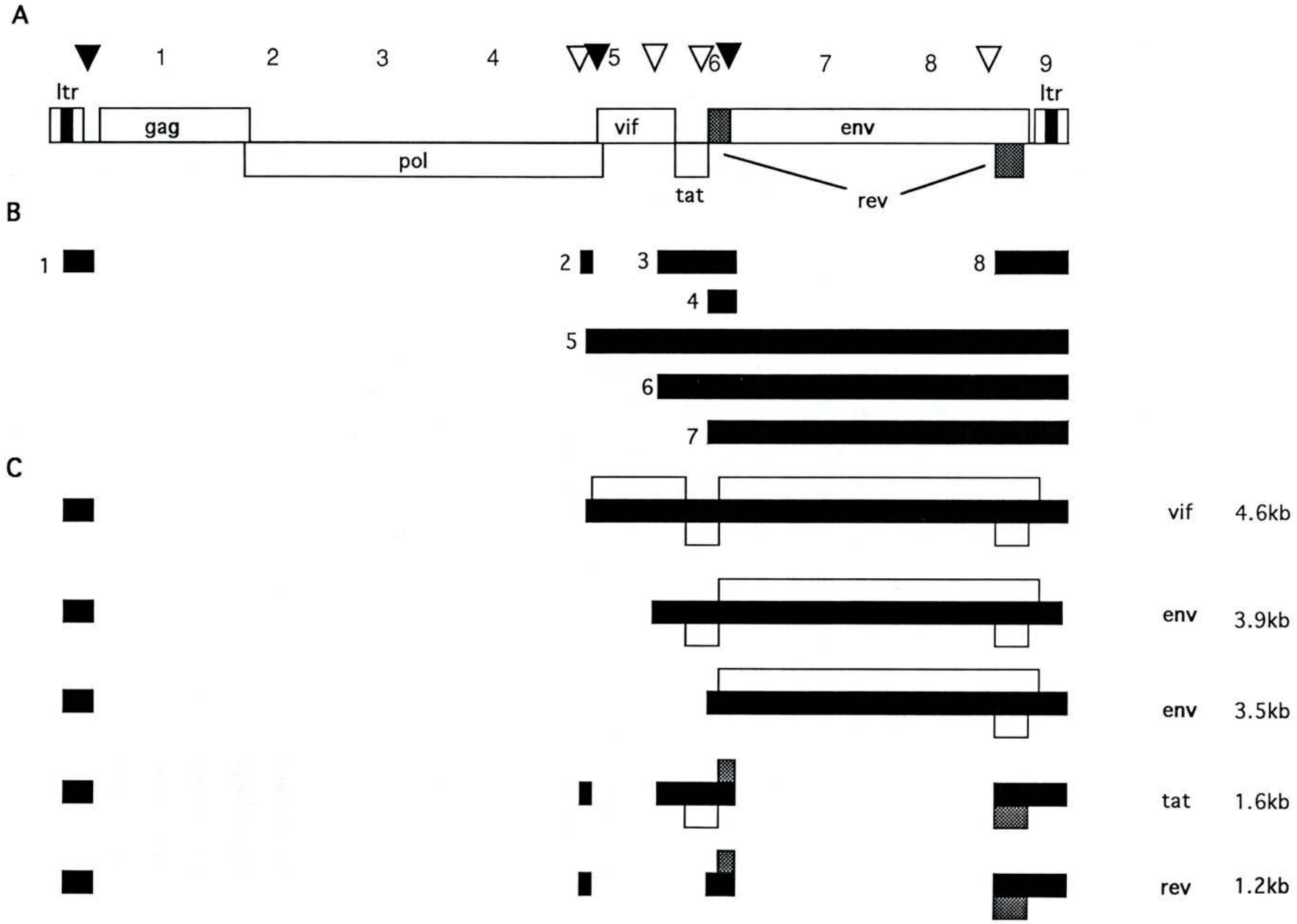
Genomic structure of Maedi Visna Virus. Numbers refer to genomic size (in kb). The bipartite gene, *rev*, is stippled. Major elucidated splice sites are depicted: filled triangles: splice donor sites, open triangles: splice acceptor sites. Position of sites based on data from Vigne *et al.*, 1987; Sargan and Bennet, 1989. Minor splice sites have been located (Sargan and Bennet, 1989): Observed variations in transcript size (for example 1.2-1.4kb *rev* mRNA) are explained by utilisation of these alternative sites.

B

Major exons. Numbering derived from Vigne *et al.*, 1987.

C

Major subgenomic mRNA species. Coding regions are depicted as open boxes. Exons 1 and 2 are non coding. Potential translation product of each species is shown, together with predicted mRNA size. Two size classes are evident: singly spliced 3.5-4.6kb RNA and multiply spliced 1.2-1.6kb RNA. Three of the mRNAs are bicistronic; there is evidence for translation of both Tat and Rev from a single multiply spliced transcript in an *in vitro* translation system (Davis and Clements, 1989). Multiply spliced transcripts with three or four exons have been reported: the presence or absence of the non-coding exon 2 may have little significance (Mazarin *et al.*, 1988 Gourdou *et al.*, 1989).


A
1
2
3
4
5
6
7
8
9
ltr
ltr
gag
pol
vif
env
tat
rev
B
1
2
3
4
5
6
7
8
C
vif 4.6kb
env 3.9kb
env 3.5kb
tat 1.6kb
rev 1.2kb

complex pattern of sub-genomic mRNAs (Vigne *et al.*, 1987; Sargan and Bennet, 1989) characteristic of lentiviruses, including HIV-1 (Muesing *et al.*, 1985), and of other 'complex' retroviruses, including HTLV-1 (Seiki *et al.*, 1985). Alternative splicing is one mechanism which allows a high proportion of the retroviral genome to encode protein: only a single set of regulatory/processing signals are required. Such economy is characteristic of RNA viruses. The basic transcript pattern of MVV is illustrated by figure 1.3. All transcripts contain common 5' and 3' sequences (Vigne *et al.*, 1987). Three size classes of mRNA are discernible; 9.4kb genomic RNA encoding Gag and Pol, 3.7-4.8kb singly spliced RNA encoding Env and Vif and 1.2 and 1.6kb multiply spliced mRNA encoding the putative regulatory proteins Rev (Mazarin *et al.*, 1988) and Tat (Davis and Clements, 1989) respectively. The complexity of the lentiviral transcription pattern (HIV-1 expresses over 20 distinct mRNA species) necessitates the presence of efficient regulatory mechanisms. In all retroviruses, *cis*-acting signals control the efficiency of alternative splicing events (see 1.13.3). However, in MVV the accumulation of transcripts is subject to temporal regulation. Specifically, viral RNA is enriched for multiply spliced transcripts early after infection; singly spliced and unspliced message accumulates at later time points (Vigne *et al.*, 1987; Sargan *et al.*, 1994). A similar biphasic pattern is observed in HIV-1 and HTLV-1 infected cells (Hidaka *et al.*, 1988; Kim *et al.*, 1989a). In HIV-1, the genes *rev*, *tat* and *nef* are early gene products, and the remaining auxiliary genes are expressed in the late phase. Regulation of mRNA accumulation allows efficient separation of regulatory/amplification and assembly phases, and may play an essential role in viral persistence. Temporal regulation of viral RNAs is a feature of a number of DNA viruses, including the herpesviruses and adenovirus, and is achieved by the presence of stage-specific promoters. A similar strategy has been proposed to have been adopted by the spuma-retrovirus subfamily (Lochelt *et al.*, 1993). In contrast, regulation in the other complex retroviruses occurs at the post-transcriptional level and is mediated by an RNA binding protein, Rev (section 1.10).

1.8 Lentivirus Auxiliary Genes

The presence of several small proteins with a regulatory or auxiliary function is characteristic of a group of retroviruses which have been termed 'complex' (Cullen, 1991). In addition to the lentiviruses, this grouping includes certain oncoviruses (prototype HTLV-1) and the spumaviruses (prototype, Human Foamy Virus: HFV) (Achong *et al.*, 1971). The extent of the

evolutionary distance between these viruses suggests that auxiliary gene products with similar functions are likely the product of convergent evolution, and are not derived from genes found in a common ancestral virus. Recombination, transduction of cellular genes ('gene capture') and duplication are all possible mechanisms for the genesis of the auxiliary genes (Myers *et al.*, 1992; Tristem *et al.*, 1992; Kubota *et al.*, 1994). The greatest array of auxiliary genes is displayed by the lentiviruses. Table 1.1 lists the complement of genes found in each of the studied lentiviral species.

Table 1.1: Lentivirus Auxiliary Genes

GENE / VIRUS GROUP	tat	rev	nef	vif	vpr	vpu	vpx
HIV-1/SIVcpz	Y-1	Y	Y	Y	Y	Y	N
HIV-2/SIVmac	Y-1	Y	Y	Y	Y	N	Y
SIVagm	Y-1	Y	Y	Y	Nc	N	Yc
SIVmnd/SIVsyk	Y-1	Y	Y	Y	Y	N	N
MVV	Y-2	Y	Na	Y	Nd	N	N
CAEV	Y-2	Y	N	Y	Nd	N	N
EIAV	Y-1	Y	N	N	N	N	N
BIV	Y-1	Y	N	Y	Ne	N	N
FIV	Y-2	Y	Nb	Y	N	N	N

Presence of each of the seven authenticated *in vivo* auxiliary gene products of lentiviruses in the genome of each phylogenetic group is illustrated. Y: gene present, N: gene not present. Additional information - **tat**: 1 - tat/TAR system, 2 - TAR independent system (see 1.9). **nef**: a - genome contains region with potential to encode sequence with degree of homology to *nef* (Sargan *et al.*, 1991): not translated in all strains; b - report of nef activity in the TM2 FIV strain (Miyazawa *et al.*, 1993), no confirmation. **vpr**: c - vpx gene of SIVagm suggested reclassification as vpr based on sequence homology (Tristem *et al.*, 1992); d - degree of homology between tat and HIV/SIV vpr in these viruses (Myers *et al.*, 1992) (1.9.3); e - BIV vpw or vpy suggested possible homologues of vpr (Gonda *et al.*, 1992): no evidence for *in vivo* translation of these ORFs.

Homologous genes do not always share extensive sequence similarity, and may be identified by function, possession of characteristic domain structure and genomic location. Two of these genes, *tat* and *rev* are common to all of these viruses. Functional homologues of *tat* and *rev* are found in the complex oncoviruses (*tax* and *rex* respectively) and of *tat* in the spumaviruses (*taf*).

Tat and *rev* are the best characterised of the regulatory/auxiliary gene class, due in large measure to the fact that functional copies of these genes are an essential requirement for HIV-1 replication (Dayton *et al.*, 1986; Fisher *et al.*, 1986; Sadaie *et al.*, 1988; Terwilliger *et al.*, 1988). Study of the remaining genes has been hampered by the lack of easily identifiable negative phenotypes. Ablation of these genes is associated with reduced, but not completely attenuated, viral replication. This phenomenon has led to these genes being termed 'auxiliary' or 'accessory', and their activities 'facilitating' as opposed to the essential *tat* and *rev*. However, the conservation of these genes implies strong selective pressure in their favour. The recent finding that attenuated auxiliary genes are associated with the failure of disease progression in certain HIV^+ individuals points to the importance of these genes in viral pathogenesis (Michael *et al.*, 1995a). Other experiments have strongly suggested that the importance of these gene products lies in the establishment and persistence of infection in the natural host environment (Kestler *et al.*, 1991; Lang *et al.*, 1993). Thus, elucidation of the functional mechanisms of the auxiliary proteins is essential for a full understanding of lentiviral pathogenesis. In this review all non-structural genes of the complex retroviruses will be termed auxiliary.

It is possible that a putative 'ancestral lentivirus' contained a basic complement of auxiliary genes (*tat*, *rev*, *vif* and possibly *vpr*). EIAV may have secondarily lost the *vif* locus. Moreover, the presence of additional complexity in the primate lentiviruses supports their classification as a distinct sub-group (see section 1.2) (Myers *et al.*, 1992). It will be interesting to identify the role of these genes in primate viral replication, and whether the non-primate viruses lack these functions, or possess compensatory activity elsewhere in their genomes. Further phylogenetic data may be derived by elucidation of the functional interchangeability of the auxiliary proteins.

A review of the information presently available concerning the lentivirus auxiliary genes follows. *In vivo* expression of the putative genes *vpw*, *vpy* and *tmx* of BIV (Garvey *et al.*, 1990) and *ttm* of EIAV (Beisel *et al.*, 1993) has yet to be demonstrated, and therefore these genes have not been included in this review.

1.8.1 Vif

Loss of function of the 23kDa Vif (**v**irion **i**nfectivity **f**actor) protein of HIV-1 is associated with a 100-1000 fold decrease in the efficiency of cell free viral transmission (Fisher *et al.*, 1987; Strebel *et al.*, 1987). The requirement for vif activity varies with cell type. Broadly, Vif is essential for replication in primary cells (restricted cells), but may be dispensable for replication in

transformed cell lines (permissive cells) (Gabuzda *et al.*, 1992a; Gabuzda *et al.*, 1994). The vif$^-$ defect is conferred upon a virus by the producer cell. Thus *vif* mutant viruses produced in restricted cells are unable to replicate in any target cell, whereas mutants produced in permissive cells can replicate in any target for at least one replicative cycle. It is assumed that permissive cells contain a factor with Vif-like activity, and/or that restricted cells possess a replication inhibition factor neutralised by Vif. Cell fusion experiments have so far failed to identify such factors (Borman *et al.*, 1995).Variations between cell lines with respect to categorisation may be responsible for divergent reports on the mechanism of Vif action.

Vif function has been shown to be required at a late stage of infection (Blanc *et al.*, 1993). The protein is present in both a soluble cytosolic form, and tightly associated with the cytoplasmic face of cellular membranes (Goncalves *et al.*, 1994). This is consistent with an effect of Vif on the assembly and maturation of virion particles. Hoglund and colleagues (1994) have demonstrated that Vif deficient particles recovered from restrictive cells have heterogeneous morphology and contain abnormal core structures. Viral protein content is also abnormal in both a quantitative and qualitative fashion. Specifically, there is a decrease in envelope (Sakai *et al.*, 1993) and polymerase gene products, and a block in proteolytic processing of the $Pr55^{gag}$ precursor molecule (Borman *et al.*, 1995; Simm *et al.*, 1995). Vif is therefore involved in the correct incorporation and assembly of viral structural proteins into the virion, and may facilitate the processing of precursor molecules. How Vif affects this process is not known. A report that Vif contains cysteine protease activity, and acts to modify the cytoplasmic domain of the transmembrane protein gp41 (Guy *et al.*, 1991) has not been confirmed (Gabuzda *et al.*, 1992b; von Schwedler *et al.*, 1993).

The block to replication of defective virions is likely to occur during viral entry as a consequence of the particle abnormalities (Courcoul *et al.*, 1995). It may involve reduced or abnormal membrane fusion, uncoating, core stability or transport following internalisation. Borman and co-workers (1995) were able to partially rescue a *vif* mutant virus by pseudotyping with murine leukaemia virus envelope, suggesting that the mutant defect is manifest in envelope mediated infection mechanisms: binding, fusion or entry. However, several groups report the unhindered entry of vif mutants into cells (Ma *et al.*, 1990). Defective viruses are reported to be unable to complete the process of reverse transcription (Sova and Volsky, 1993; von Schwedler *et al.*, 1993). This may be manifest as an indirect effect. Completion of reverse transcription may

occur within a partially uncoated core structure: disruption of this structure is likely to impair the enzymatic process. Thus, it appears that Vif modulates particle maturation in such a way that viral infectivity is enhanced by the stimulation of efficient reverse transcription.

A hydrophobic carboxy-terminal region of Vif may be involved in membrane interactions. This region contains a motif conserved amongst almost all lentiviral Vif proteins, with the consensus sequence SLQXLA (Oberste and Gonda, 1992). Conservation of this motif points to a degree of functional conservation of the Vif protein. Loss of Vif function is associated with a similar phenotype to that of HIV-1 in HIV-2 (Michaels *et al.*, 1993), SIV_{mac} (Gibbs *et al.*, 1994) and FIV (Tomonaga *et al.*, 1992). The Vif proteins of SIV_{mac} and HIV-1 demonstrate functional reciprocity in the HUT78 cell line (Simon *et al.*, 1995); this property is not shared by the Vif proteins of BIV, FIV and MVV. This may be due to a failure of the non-primate lentivirus proteins to interact correctly with the HIV-1 viral proteins which are the putative targets for Vif. However, *vif* mutant HIV-1 and HIV-2 viruses show differential replication in certain cell lines, suggesting that they have different requirements for cellular factors (Reddy *et al.*, 1995b). The 29kDa product of the MVV *vif* gene has been shown to localise to the cytoplasm of infected cells, although no association with cellular membranes was found (Audoly *et al.*, 1992). Vif is essential for efficient replication of CAEV in primary macrophages (Harmache *et al.*, 1995a). Deletion of CAEV Vif is associated with a defect in the late stage of virus production; however, these authors did not identify a reduction in viral infectivity. Although this might suggest a divergence in mechanism between HIV-1 and CAEV Vif proteins, the uncertainty over the distribution of Vif-permissive and restrictive cell lines for non-primate lentiviruses makes interpretation of this data difficult.

1.8.2 Nef

The *nef* gene is found only in primate lentiviruses. The 25-27kDa product is modified by myristoylation of an amino-terminal glycine residue, which results in association of this cytoplasmic protein with the plasma membrane of infected cells (Franchini *et al.*, 1986; Yu and Felsted, 1992). The function of Nef is one of the most controversial aspects of lentivirus molecular biology. Early studies proposed a role for Nef in the repression of transcription from the HIV-1 proviral LTR (Ahmad and Venkatesan, 1988; Niederman *et al.*, 1989), hence the term nef (**n** **e**gative **f**actor). However, subsequent analysis failed to confirm these results (Kim S. *et al.*, 1989), and instead identified an enhancement of viral replication kinetics in a wide range of host cells in the presence of a functional *nef* gene (de Ronde *et al.*, 1992; Terwilliger *et al.*, 1991).

This effect may be attributable to an increase in the intrinsic infectivity of progeny viral particles produced in the presence of Nef (Miller *et al.*, 1994). Nef has not been found within the virion, suggesting that it may act through post-translational modifications to the viral structural proteins: reminiscent of the role of Vif. Recently, two groups have reported that proviral DNA synthesis in cells infected with *nef* deleted (Δnef) HIV-1 proceeds 5-10 fold less efficiently than wild type (Aiken and Trono, 1995; Schwartz *et al.*, 1995). The apparently normal viral RNA and protein content, and normal reverse transcriptase activity of isolated particles suggests that the Δnef defect is due to the existence of a suboptimal environment for DNA synthesis. Inappropriate internalisation or uncoating of the viral core due to the absence of a Nef mediated modulation might explain this phenomenon. A highly conserved proline repeat motif with the sequence $(PXX)_4$ is required for enhancement of infectivity, and may bind a cellular serine kinase (Sawai *et al.*, 1994). Recruitment of a kinase by Nef may result in modifications to the viral structural proteins.

A second biological function of Nef is the specific removal of cell surface CD4 molecules by endocytosis into early endosomes, with subsequent degradation (Garcia and Miller, 1991; Anderson *et al.*, 1993; Aiken *et al.*, 1994). Removal of surface receptor may prevent deleterious viral superinfection (Benson *et al.*, 1993), and aid dispersal of progeny virions. However, mutational analysis has demonstrated dissociation of this activity from viral infectivity enhancement (Goldsmith *et al.*, 1995).

Surface CD4 is involved in signal transduction resulting in activation of T lymphocytes by antigen stimulation. Downregulation of CD4 may alter the activation state of cells. There are directly contradictory reports as to the affect of Nef on cell activation (Bandres and Ratner, 1994; Skowronski *et al.*, 1993). A possible explanation for this lies in the findings of Baur *et al.* (1994), that Nef promotes activation when expressed at the cell surface, but is inhibitory when present in the cytoplasm. This effect is presumably mediated by interactions with different sets of cellular proteins. The influence of such activities on viral replication and pathogenesis is unclear.

The *nef* gene of SIV_{mac} appears to be functionally analogous to HIV-1 nef. There is sufficient conservation between the proteins to allow the functional substitution of the SIV_{mac} *nef* gene with that of HIV-1 (Sinclair *et al.*, 1995). Studies with SIV_{mac} infection of rhesus monkeys (*Macaca mulatta*) have demonstrated the essential nature of Nef for viral spread and pathogenicity in infected animals (Kestler *et al.*, 1991). Moreover, the most successful vaccines developed to date against SIV infection have been live viruses with attenuating *nef* mutations

(Daniel *et al.*, 1992). Subtle effects of Nef on infectivity and T lymphocyte physiology, difficult to interpret *in vitro*, may be of great importance *in vivo*.

Nef specific transcripts may represent 90% of mRNAs expressed early in infection (Klotman *et al.*, 1992). Such high level expression might have significance for early antiviral immune responses. Nef contains several epitopes recognised by cytotoxic T lymphocytes, and these are found in the majority of HIV-1 infected individuals (Culmann *et al.*, 1989; Hadida *et al.*, 1992). The significance of these cells is unknown.

1.8.3 Vpr

Viral Protein R (Vpr), is the 12-15kDa product of the R ORF common to all primate lentiviruses (except possibly SIV_{agm}, see table 1.1) (Wong-Staal *et al.*, 1987). The phenotype associated with Vpr is host cell dependent. The emerging consensus is that Vpr conveys an advantage in terms of rapidity of replication and higher progeny virus titres in primary cells (Westervelt *et al.*, 1992; Balotta *et al.*, 1993; Connor *et al.*, 1995). Lack of functional Vpr is associated with moderate to complete attenuation of viral replication in monocytes and macrophages (Balliet *et al.*, 1994). *Vpr* deletion has no significant effect on viral replication in immortalised cell lines (Hattori *et al.*, 1990). Vpr may be dispensable for growth in all cell types in HIV-2 and SIV_{mac} (Gibbs *et al.*, 1994); this may be due to compensatory activity of the Vpx protein (see below).

Vpr is unique amongst HIV-1 auxiliary proteins in being incorporated into the virion (Yuan *et al.*, 1990), and is present in approximately equimolar quantities to Gag (Paxton *et al.*, 1993). Interaction with the carboxy-terminal Gag protein p6 is both necessary and sufficient for incorporation of Vpr into nascent virions (Kondo *et al.*, 1995).

Vpr locates specifically to the nuclear matrix and perinuclear regions in transfected cells (Lu *et al.*, 1993). Nuclear localisation may be a function of a leucine/isoleucine rich motif in the central region of Vpr (Zhao *et al.*, 1994), and may be mediated by interaction with a cellular protein.

Several functional activities are associated with Vpr. Incorporation in the virion strongly suggests an involvement in the early phase of infection. One hypothesis is that the nucleophilic Vpr protein ensures nuclear import of the replicative pre-integration complex, which allows replication in non-dividing cells such as macrophages (Heinzinger *et al.*, 1994).

Post integration events may also be influenced by Vpr. Purified, recombinant Vpr can

reactivate HIV-1 expression from latently infected cell lines and induce an increase in permissiveness to HIV replication amongst a range of both transformed and primary cells (Levy *et al.*, 1995). Cohen and co-workers (1990) demonstrated modest (3-fold) activation of HIV-1 LTR and heterologous promoters by Vpr. The amino-terminal region of Vpr contains a motif with some homology to the acidic activation domain of a range of *trans*-activating factors, including MVV Tat (1.9.2). By analogy with these proteins, the activity of Vpr is probably mediated by interaction with cellular factors. Vpr *trans*-activation of the viral promoter may have significance at periods during infection when Tat is limiting, especially immediately post integration. Alternatively, Vpr mediated *trans*-activation of cellular genes may be responsible for the ability of Vpr to alter the state of cells, with consequential increases in permissiveness for viral replication. Vpr can inhibit proliferation of a wide range of cells, including T lymphocytes, by inducing arrest in the G_2 phase of the cell cycle (Rogel *et al.*, 1995), and induce differentiation in others (for example the rhabdomyosarcoma cell line TE671, Levy *et al.*, 1993). HIV-1 clones are able to establish persistent infections *in vitro* only in the absence of a functional *vpr* gene (Nishino *et al.*, 1991; Planelles *et al.*, 1995), suggesting that Vpr impairs the multiplication or viability of infected cells.

The *in vivo* functional significance of such activity is uncertain. Arrest in G_2 may establish a suitable milieu for viral replication, and prevent the re-entry of cells into G_0 quiescence. Host cell differentiation is linked to increased viral permissiveness (Rich *et al.*, 1992). Exogenous Vpr may establish a reservoir of permissive cells at an initial stage of infection before its effects are reduced by humoral immunity. Vpr is present within the serum and cerebro-spinal fluid of AIDS patients (Levy *et al.*, 1994). Dysregulation or differentiation of uninfected cells may contribute to pathology: loss of muscle precursor populations for example, may explain AIDS associated wasting (Levy *et al.*, 1995). A similar effect on immune cell precursors may contribute to depletion of mature immune cells.

Studies with mutated *vpr* genes in SIV_{mac} infection have reported that *vpr* is dispensable for the establishment of infection and pathogenicity (Gibbs *et al.*, 1995; Hoch *et al.*, 1995), although some delay in progression to disease may occur (Lang *et al.*, 1993). Hoch and co-workers did, however, indicate that there might be a more vigorous humoral immune response against the virus in the absence of *vpr*. Cells chronically infected with Δ*vpr* virus might be better targets for the immune system. This, in turn, could allow the immune system to contain the infection for a longer period. The relative contribution of each function of Vpr may vary according to host cell type.

1.8.4 Vpx

A 14-16 kDa protein has been identified as the product of the X ORF of the HIV-2/SIV_{mac}/SIV_{sm} group of lentiviruses (Franchini *et al.*, 1988). *Vpx* has significant homology with *vpr* and may have arisen due to a gene duplication event in the relatively recent evolutionary past (Tristem *et al.*, 1990). This suggests the possibility of a duplicative or overlapping functional role for these genes; a hypothesis strengthened by the fact that, like Vpr, Vpx is packaged into mature virions (Henderson *et al.*, 1988, Yu *et al.*, 1988). Furthermore, a similar phenotype is associated with ablation of Vpr and Vpx expression: similar cell type specific effects are observed (Hu *et al.*, 1989; Marcon *et al.*, 1991;Yu *et al.*, 1991; Gibbs *et al.*, 1994). Gibbs *et al.* (1994) found that Δvpx SIV_{mac} viruses demonstrated a significantly greater degree of attenuation than Δvpr mutants: deletion of both *vpr* and *vpx* severely attenuated viral replication. Infection of rhesus monkeys with *vpx* deleted SIV_{mac239} virus resulted in a reduction in viral load but did not prevent progression to AIDS (Gibbs *et al.*, 1995).

The nature of the attenuation of replication by *vpx* deletion is unknown. Both the presence of Vpx within the virion and its localisation within the cytoplasm of infected cells (Kappes *et al.*, 1993) suggest an involvement in the early events of replication. A 5-10 fold reduction in DNA synthesis within a single replicative cycle is associated with loss of Vpx function (Kappes *et al.*, 1991). A role for Vpx in reverse transcription is therefore possible. Binding of purified Vpx to polyethenoadenylic acid (Henderson *et al.*, 1988) is suggestive of a role in stabilising RNA and DNA interactions during reverse transcription.

Further study is required to demonstrate the similarity of Vpx and Vpr. It is possible that divergence is taking place, with novel functions co-existing with overlapping ones. The relative contributions of Vpr and Vpx in HIV-2/SIV infections could vary in different viral backgrounds and host cell types. Vpx may be 'dominant' in the SIV_{mac239} strain used by Gibbs and co-workers (1994).

1.8.5 Vpu

Viral protein U (Vpu) is a 16kDa protein unique to the HIV-1/SIV_{cpz} group of primate lentiviruses. Vpu is an oligomeric transmembrane phosphoprotein (Strebel *et al.*, 1989; Maldarelli *et al.*, 1993), with two elucidated functions.

Replication of viruses deficient for Vpu expression is associated with decreased

production of progeny virions, and a concomitant accumulation of viral proteins within infected cells (Strebel *et al.*, 1988; Terwilliger *et al.*, 1989). The Vpu mutant phenotype is also characterised by the presence of abnormal viral particles, and by aberrant budding from intracytoplasmic membranes (Klimkait *et al.*, 1990). Thus, Vpu facilitates the correct assembly and/or final release of virions. The affect of ablation of Vpu on viral replication is dependent on host cell type: replication is strongly attenuated in certain transformed cell lines and in primary monocytes/macrophages (Balliet *et al.*, 1994; Yao *et al.*, 1992), but little affected in others, for example COS-7 cells (Gottlinger *et al.*, 1991). Vpu can mediate the enhanced release of capsids containing Gag-Pol precursor proteins from divergent retroviruses, including MVV and Moloney murine leukaemia virus (Gottlinger *et al.*, 1993); activity is therefore unlikely to involve specific interactions with capsid proteins. Vpu has biochemical and structural homology with several ortho- and para-myxovirus proteins which are involved in virion release, including influenza A virus M2. M2 has been shown to act as an ion channel by forming multimeric pore structures within Golgi membranes (Pinto *et al.*, 1992). Thus, by analogy, Vpu may alter luminal conditions with respect to pH or ion balance which in turn leads to modification of the capsid precursor proteins to ensure efficient assembly and release. The putative modification may also be mediated by cellular factors, obviating the need for Vpu in other retroviruses, or in certain host cell types.

A second function of Vpu has recently been elucidated. During export from the cell, the viral precursor envelope protein gp160 is trapped within the endoplasmic reticulum (ER) by the formation of complexes with its ligand molecule, CD4, preventing efficient processing and export (Jabbar and Nayak, 1990). Expression of Vpu, which localises to the perinuclear membrane compartments within the cytoplasm (Klimkait *et al.*, 1990), alleviates the block in gp160 processing by inducing the degradation of CD4 (Willey *et al.*, 1992). The half life of CD4 molecules in the presence of Vpu is reduced from six hours to twelve minutes (Lenburg and Landau, 1993). Conformational changes in CD4 triggered by Vpu binding might be responsible for degradation, perhaps by entry of the altered protein into the ER pathway for the proteolysis of misfolded proteins (Vincent *et al.*, 1993; Bour *et al.*, 1995).

The functional significance of the two roles of Vpu are incompletely understood. They appear to be mediated as independent events (Schubert and Strebel, 1994). In particular, cells of the monocyte/macrophage lineage express little CD4 and display budding of particles from intracytoplasmic vesicles in the absence or presence of Vpu (Orenstein *et al.*, 1988). Yet viral

replication in macrophages is particularly susceptible to loss of Vpu function. It is possible that this is due to other, as yet, uncharacterised functions of Vpu.

1.9 Tat

All lentiviruses encode a small, regulatory protein, Tat, which mediates *trans*-activation of the viral U3 promoter. There is evidence to suggest that lentiviruses differ in both the mechanism of *trans*-activation and in their requirement for Tat activity. Broadly, lentiviruses divide into two groups with respect to Tat: those for which Tat is an absolute requirement, correlating with high *trans*-activating activity (HIV-1, HIV-2, SIV, BIV and EIAV), and those (MVV, CAEV, FIV) where Tat may be dispensable for replication in culture, correlating with low level *trans*-activation. This division may be due to the characteristics of basal (- tat) transcription, such that in the latter group basal transcription may be sufficient to support replication, and may reflect fundamental differences in the mechanism of *trans*-activation.

1.9.1 HIV-1 Tat

HIV-1 tat (tat-1) is an 86 residue, highly basic protein encoded by two exons. Tat-1 has a modular structure, with a number of protein domains responsible for mediating different activities (figure 1.4a). Modular structure is a characteristic of transcriptional activators (Ptashne, 1988). A highly basic central motif is required for target binding *in vitro* (Calnan *et al.*, 1991), and is also responsible for targeting of Tat-1 to the nucleus, and in particular, the nucleolus of infected cells (Hauber *et al.*, 1989). The 47 amino terminal residues, comprising an acidic terminal region, the cysteine and 'core' motifs, are required for function, and form the 'activation' or 'effector' domain of Tat (Rice and Carlotti, 1990).

The basal activity of the HIV-1 promoter is low in most cells (Jones *et al.*, 1986). Tat-1 induces transcription from the promoter by 40-3000 fold, depending on cell type (Arya *et al.*, 1985; Sodroski *et al.*, 1985). This activity is dependent on binding to TAR (**t**rans-**a**ctivation **r**esponse element), an RNA stem-loop structure present in all nascent viral transcripts. Tat/TAR interaction is essential for HIV-1 replication (Fisher *et al.*, 1986). TAR is located within the R region of the LTR, immediately downstream of the transcriptional initiation site (bases +1 to +44). A highly conserved trinucleotide pyrimidine bulge within TAR is the site of Tat binding (Dingwall *et al.*, 1990). The bulge may induce a bend in the RNA helix to allow Tat access to the major groove,

Figure 1.4: Structure of the MVV and HIV-1 Tat Proteins

A

Domain organisation of the Tat proteins of HIV-1 and MVV. Delineating residue position of each domain are shown. The putative activation domain of each protein is marked. A cysteine rich region provides the single region of amino acid homology. NH2: amino terminus, COOH carboxy terminus.

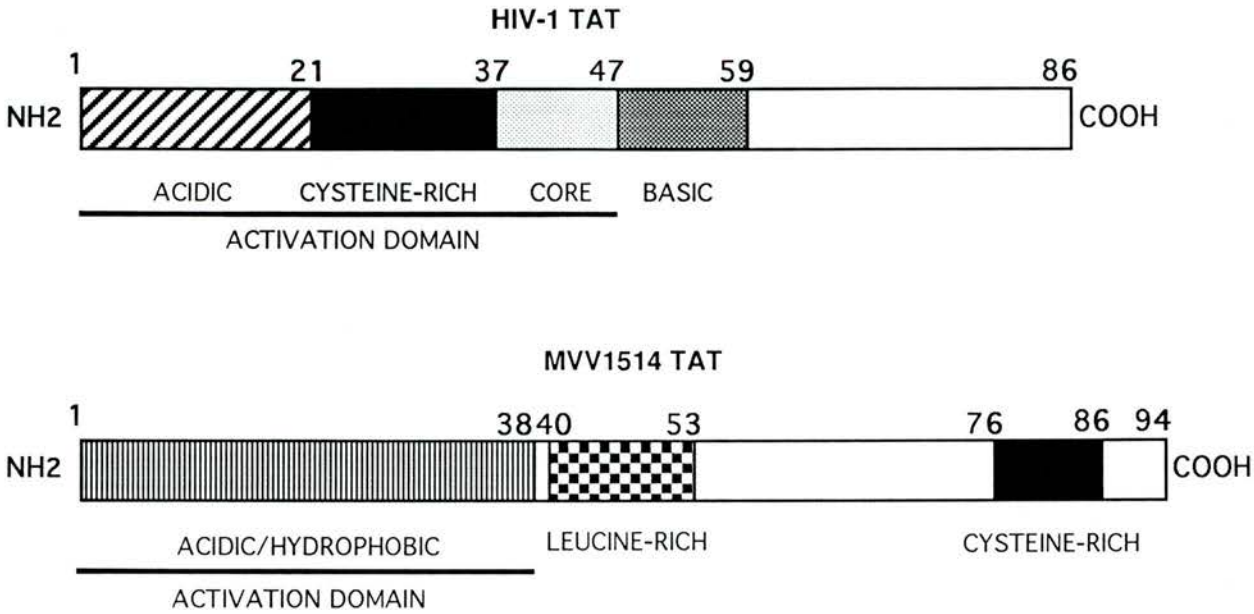
B

Amino acid homology within cysteine-rich domains. Top: HIV-1 Tat sequence, residue 11-30. Bottom: MVV Tat sequence, residues 65-81. Identical residues are shown in **bold**. Gap in sequence are depicted by a dash.

C

Alignment of sections of the putative activation domains of the transcriptional *trans* activators, herpes simplex virus type 1 (HSV-1) VP16, and MVV Tat (from Carruth *et al.*, 1994 The amino terminal portion of the HIV-1 Vpr protein, which may have *trans*-activating activit (section 1.8.3) is also shown. The carboxy-terminal proximal regions display greatest sequenc similarity. **Bold**: acidic residues, *italics*: hydrophobic residues.

A



B

HIV-1 Tat

11 **W K H** P **G** S Q P K T A C T T **C** Y **C** K K **C** 30

W K H K **G** A A V R R N - - - **C** G **C** R L **C**

65 81

MVV Tat

C

HSV VP16 457 H *L* D G **E D** *V* A *M* A H A **D** A *L* **D D** *F* **D** *L* - **D** *M L* 480

MVV1514 TAT 3 **E** *V* P R R Q P G G *L* - *V* **E** A **E** G *V F* Q *F Y* **E D** *W* 26

HIV-1 VPR 4 Q A P **E D** Q P G R **E** - P H N **E** *W* T *L* **E** *L L* **E E** *L* 27

resulting in a stable interaction.

Tat-1 activity leads to an increase in the steady state level of LTR directed transcripts (Peterlin *et al.*, 1986). However, the precise mechanism of Tat activity is incompletely understood and remains controversial. Analysis of viral RNA produced in the absence of Tat-1 has shown that most transcripts terminate close to the initiation site, consisting largely of the TAR structure (Feinberg *et al.*, 1991). Tat-1 function is associated with an increase in the number of full-length transcripts with little change in the number of short ones (Kao *et al.*, 1987). Expression of the transcription factor TFIIF, believed to have a role in increasing polymerase processivity, obviates the requirement for Tat-1 (Kato *et al.*, 1992). The poor efficiency of elongation of the basal transcriptional complex is a result of the assembly of a poorly processive complex in the absence of Tat (Kessler and Matthews, 1992). This is presumably mediated by elements within the LTR. Thus, the HIV-1 LTR has been described as an 'attenuated' promoter (Jones and Peterlin, 1994), and Tat-1 as an 'RNA sequence specific processivity factor' (Cullen, 1991).

There is also evidence for a second mechanism of Tat-1 activity: direct increase in the rate of transcription initiation from the viral LTR (Kao *et al.*, 1987; Rice and Matthews, 1988). This activity is almost completely analogous to the action of conventional transcriptional activators (reviewed in Roeder, 1991), and suggests that Tat-1 can co-operate with cellular transcription factors to maximise the rate of initiation. The mechanism of increased initiation is not fully understood. Binding of Tat-1 to the **T**ATA **b**inding **p**rotein (TBP), a component of the TFIID basal transcription complex, has been demonstrated, and is necessary for Tat-1 activity (Kashanchi *et al.*, 1994; Zhou and Sharp, 1995). Tat-1 binding was found to enhance TFIID interactions with the promoter.

Laspia and co-workers (1989) have provided evidence to support both models of Tat function. Indeed, it is likely these events are linked, with the formation of the transcriptional complex influencing its subsequent activity. A full appreciation of the activity of Tat will await the evaluation of the role of cellular co-factors. A strong candidate for a Tat co-activator has recently been reported. This is a protein kinase (TAK: **t**at **a**ssociated **k**inase) which has been shown to interact with the activation domain of Tat-1 and Tat-2 (Herrmann and Rice, 1995). The ability of mutant Tat proteins to interact with TAK was found to correlate with *trans*-activation ability. A known substrate for TAK is RNA polymerase II. Phosphorylation has been proposed to trigger the transition from the initiation to the elongation phase of transcription (Dahmus and Dynan, 1992). Several other Tat and TAR binding proteins have been described (reviewed in Antoni *et*

al., 1994): in most cases the functional significance of interaction remains to be determined.

A bimodal mechanism for Tat-1 activity is suggested by the data presently available. Thus, Tat may upregulate the rate of formation of initiating ribonucleoprotein complexes by means of direct interaction with specific components, for example TBP. Subsequently, binding of Tat-1 to the nascent RNA may promote elongation over initiation by recruiting TAK to the complex.

Whilst the reductionist approach has yielded some success in delineating Tat function, it may be insufficient to explain the full role of this protein. In particular, the increase in the steady state levels of viral mRNA resulting from Tat activity is insufficient to account fully for the positive effect of Tat on protein expression and viral replication (in HIV-1: Cullen, 1986; Feinberg *et al.*, 1986) (in MVV: Gdovin and Clements, 1992). Tat may therefore possess a post-transcriptional role. Experiments studying HIV-1 Tat activity in *Xenopus* oocytes demonstrated the requirement for Tat to 'activate' transcripts to allow efficient translation (Braddock *et al.*, 1989). This observation has not been repeated in human cells, however (Chin *et al.*, 1991). Huang and colleagues (1994) have observed reduced infectivity and cytopathicity of HIV-1 containing a deleted *tat* gene but with a compensatory *trans*-activating function, suggesting a discrete role for Tat-1 in viral infectivity.

The Tat/TAR axis is preserved by HIV-2 and SIV, and by the distantly related BIV and EIAV. The Tat proteins of HIV-1, HIV-2 and SIV_{mac} can functionally complement one another (Viglianti and Mullins, 1988; Tong-Starksen *et al.*, 1993). Whilst the BIV Tat/TAR system possesses many of the elements of *trans*-activation found in the primate lentiviruses (Pallansch *et al.*, 1992; Carpenter *et al.*, 1993) EIAV is more diverse. There is no bulge within the TAR of EIAV (Carvalho and Derse, 1991), and the Tat protein (eTat) has a simpler structure, with an activation domain consisting solely of the conserved core motif (Carroll *et al.*, 1991). Although eTat cannot functionally substitute for Tat-1, a chimaera consisting of eTat core and Tat-1 basic motifs can direct weak *trans*-activation of the HIV-1 promoter (Derse *et al.*, 1991). This suggests that these proteins function by interacting with the same cellular components.

1.9.2 MVV Tat

MVV Tat (vTat) is the prototype of the second group of *trans*-activators. MVV Tat is a 10kDa protein encoded by a single exon and possesses a distinct domain structure from Tat-1 (Davis and Clements, 1989; figure 1.4a). The only homologous region is the cysteine rich motif

(figure 1.4b). The relatively modest *trans*-activating ability of vTat (5-30 fold induction)(Hess *et al.*, 1989; Gourdou *et al.*, 1989) is partially due to high basal activity of the MVV LTR (Hess *et al.*, 1985; Davis and Clements, 1989). The LTR may thus be sufficiently processive in its own right. There is no TAR-like region in the LTR. Instead, vTat activity may require sequences within the LTR enhancer region, in particular the TATA box proximal AP-1 site and the neighbouring AP-4 site found in the 1514 viral strain (Hess *et al.*, 1989; Gdovin and Clements, 1992). There is no consensus AP-1 site in the EV-1 MVV isolate (Sargan *et al.*, 1995): instead an uncharacterised cellular factor binds an overlapping site (Sutton *et al.*, 1995). The effect of this on vTat activity in this virus is unknown.

There is no evidence to suggest that vTat binds directly to DNA (Gdovin and Clements, 1992; Neuveut *et al.*, 1993). vTat may thus function by an indirect mechanism. Other viral *trans*-activators function in this manner, by modulating the activity of cellular factors to recruit them to the viral promoter. The adenovirus E1A , herpes simplex virus VP16 (reviewed by Jones *et al.*, 1988) and HTLV-I Tax proteins are examples (Wagner and Green, 1993).

The transcriptional factors Fos and Jun form a heterocomplex which can bind at the TATA box proximal AP-1 site of MVV (Shih *et al.*, 1992). A role for these factors in vTat *trans*-activation is suggested by the failure of Jun deficient F9 teratocarcinoma cells to support vTat function, and by the finding that vTat elevates the expression of Jun in transfected cells (Neuveut *et al.*, 1993). Thus, vTat may directly interact with Jun and Fos, altering their binding specificities to encourage interaction with the viral promoter. This is analogous to the activity of Tax, which binds a subset of the bZIP (basic region-leucine zipper domain) cellular transcription factors, including CREB and CREM (Adya *et al.*, 1994), and stabilises their dimerisation (Baranger *et al.*, 1995; Perini *et al.*, 1995).

A vTat/GAL 4 DNA binding domain chimaera has been shown to direct activation via the recognition site for GAL 4 (Carruth *et al.*, 1994). The full length vTat protein demonstrated characteristically modest *trans*-activating ability in this system (2-4 fold). The 38 amino-terminal residues of vTat were found to have potent activity (600 fold *trans*-activation) if directly fused to GAL4, and are suggested to form an activation domain. This region of vTat has a degree of homology with the acidic activation domains of a number of transcriptional *trans*-activators. The aligned hydrophobic residues and overall negative charge of this domain is demonstrated by herpes simplex virus VP16 and by the Vpr protein of primate lentiviruses (figure 1.4b) (see section 1.8.3). Acidic activation domains are implicated in binding to basal transcription factors,

including TBP (Greenblatt *et al.*, 1994). The ability of Tat-1 to compete specifically with chimaeric vTat/GAL4 in this system (Carruth et al., 1994), demonstrates that the activation domains of these proteins may interact with a common cellular co-factor(s); TBP is a strong candidate. This activity may be common to both groups of lentiviral Tat proteins.

A possible model for vTat function is suggested. In quiescent cells, low concentrations of vTat are effectively repressed by an intramolecular interaction with a leucine rich negative regulatory element (residues 39-53) (Carruth *et al.*, 1994). On cell activation or differentiation, elevated levels of Fos and/or Jun bind to the leucine rich motif of vTat (these factors possess a leucine zipper motif), inducing conformational changes which release the activity of the activation domain. Furthermore, binding to Fos/Jun directs vTat to promoters containing AP-1 sites, including the proviral LTR. vTat may function by both stabilising Fos/Jun dimerisation and binding to DNA, and by direct interaction with other components of the basal transcription complex, including TBP.

Several features of MVV transcription and Tat biology are shared by CAEV and FIV. Both of these viruses have promoters with high basal activity and the putative Tat proteins possess moderate *trans*-activating ability (2-8 fold) (Sparger *et al.*, 1992; Saltarelli *et al.*, 1993), and act through AP-1 and AP-4 sites located within the U3 region (Hess *et al.*, 1986). The domain structure of vTat is shared by the CAEV protein, enabling these proteins to functionally complement one another (Saltarelli *et al.*, 1993). Harmache *et al.* (1995b) found that *tat* is dispensable for viral replication both *in vitro* and during experimental infection of goats. FIV tat (ORF A) has been shown to be dispensable for growth in transformed cell lines, although deletion of *tat* is associated with a severely impaired phenotype in primary lymphocytes (Tomonaga *et al.*, 1993). It seems probable that the role of Tat in these viruses is not primarily as an LTR *trans*-activator.

1.10 Rev

Sodroski and co-workers (1986) observed that mutations around the HIV-1 *tat* second exon, which could not be complemented by exogenous Tat, resulted in attenuated structural gene expression without affecting tat expression. This mutant phenotype mimics the early phase of productive infection (1.7) suggesting that a gene responsible for temporal regulation was defined by the mutation. The gene was identified and named *art* (**a**nti-**r**epressor ***t**rans*-activator),

due to the activity of the gene product in relieving repression of structural gene expression. A second group also located this gene, although they suggested the name *trs* (***t****rans*-**r**egulator of **s**plicing) because of a proposed role in the inhibition of splicing (Feinberg *et al.*, 1986). The actual effect of the gene was discovered when it was shown that *art/trs* defective proviruses failed to accumulate cytoplasmic structural gene (9 and 4kb) mRNAs, but that total cellular levels of RNA species were essentially unchanged in the presence or absence of *art/trs* (Malim *et al.*, 1988; Arrigo *et al.*, 1989; Hammarskjold *et al.*, 1989). Complementation with a functional gene restored structural gene expression by relieving the block to cytoplasmic expression of the cognate mRNA. The name *rev* (**r**egulator of **e**xpression of **v**iral proteins) has subsequently been adopted (Gallo *et al.*, 1988).

Rev is a post-transcriptional *trans*-activator of structural gene expression and is essential for productive infection (Sadaie *et al.*, 1988). Its effect is mediated via the **R**ev **R**esponse **e**lement (RRE), a region of RNA secondary structure within the *env* gene (Malim *et al.*, 1989c). This is spliced out of the rev independent 2kb mRNAs. The RRE is present in the full length (9kb) *gag/pol* and singly spliced (4kb) *env/vpu*, *vif* and *vpr* transcripts in HIV-1; expression of these transcripts is Rev dependent (Sodroski *et al.*, 1986; Knight *et al.*, 1987; Schwartz *et al.*, 1991; Garret *et al.*, 1991). Rev accumulation during infection results from the action of Tat on viral transcription. Transcripts are spliced to completion, or retained and degraded in the nucleus, in the absence of Rev. Once a sufficient level of activity is reached, Rev overcomes the effect of inhibitory sequences present in the mRNA encoding the structural proteins by one or more of the following proposed mechanisms: inhibition of splicing, promotion of nucleocytoplasmic export, increasing stability and/or translatability of these transcripts (Rosen *et al.*, 1988; Chang and Sharp, 1989; Emerman *et al.*, 1989; Felber *et al.*, 1989; Arrigo and Chen, 1991; Pomerantz *et al.*, 1992). Direct binding and oligomerisation of Rev on the RRE is necessary, but not sufficient for function, which also requires interaction with one or more cellular co-factors (Daly *et al.*, 1989; Berger *et al.*, 1991; Malim *et al.*, 1991). Rev therefore initiates the expression of structural proteins, acting as a switch between early and late phases of the viral replicative cycle.

A *rev* gene is present in the genomes of all lentiviruses, and a homologous gene, *rex*, is encoded by the HTLV-1 oncovirus group (Inoue *et al.*, 1986). Rex also acts to facilitate the cytoplasmic accumulation of incompletely spliced RNAs (Hidaka *et al*, 1988). This activity is essential for viral replication (Rosenblatt *et al.*, 1988) and is mediated by a structured region within the viral LTR, the Rex responsive element (RxRE) (Hanly *et al.*, 1989).

1.11 Rev Biochemistry: Definition of Functional Domains

Rev/Rex proteins are characteristically small, nuclear phosphoproteins. The size range of these proteins is from 82 residues (SIV_{agm}) to 189 residues (HTLV-I). Most Rev/Rex proteins migrate anomalously slowly on SDS-PAGE gels, due to the high content of basic residues and/or to post-translational modifications. Considerable progress has been made towards a biochemical characterisation of HIV-1 Rev, the prototypic protein of the family, and of the Rex protein of HTLV-1. Analysis of domain structure is of importance to facilitate elucidation of functional mechanisms.

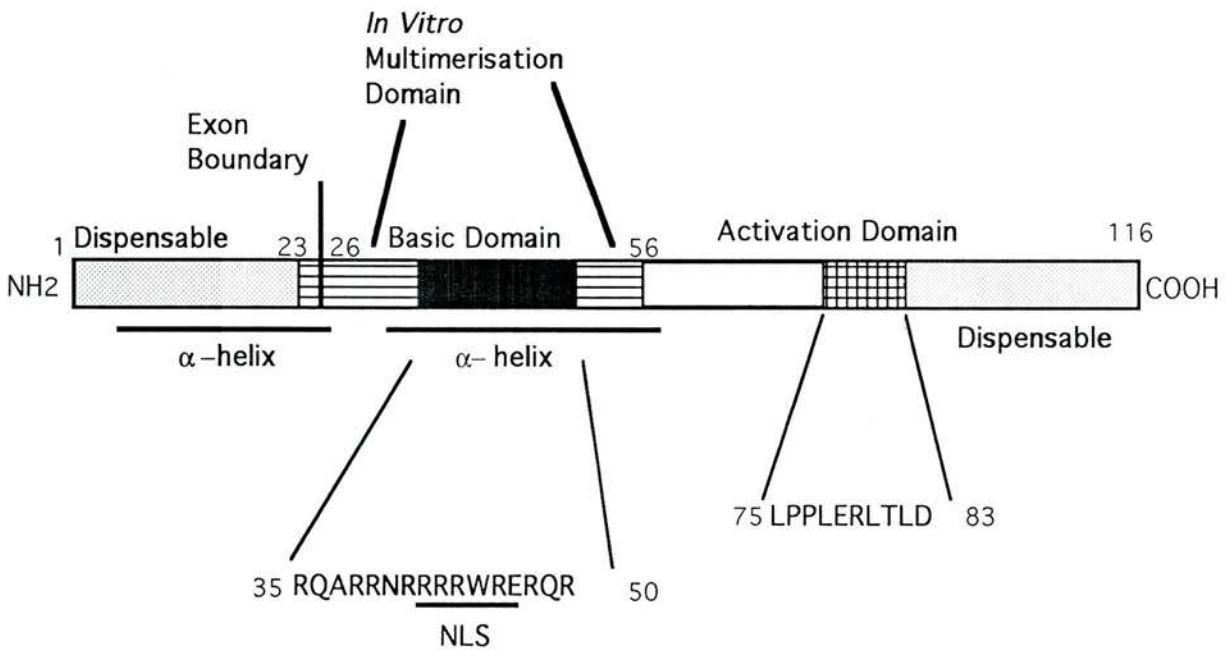
HIV-1 Rev consists of 116 amino acid residues, and is encoded by a series of multiply spliced transcripts (Schwartz *et al.*, 1990). The first coding exon is located in the centre of the genome, between *pol* and *env*, and contributes the 24 amino-terminal protein residues. The second exon, situated at the 3' end of *env*, but in a different reading frame register, encodes residues 25-116 (Sodroski *et al*, 1986). The protein migrates on SDS-PAGE gels with an apparent molecular weight of 19kDa (Knight *et al.*, 1987). Extensive mutational analysis has led to the definition of two major functional domains within HIV-1 Rev (figure 1.5). The first consists of ~40 amino terminal residues, and is characterised by an arginine-rich central core. This motif (residues 34-50) functions both as a nucleolar localisation signal, and as the RNA sequence-specific binding element. Less distinct flanking sequences are essential for *in vitro* multimerisation of the Rev protein on its target RNA. Mutations within this amino terminal domain typically reveal a recessive negative phenotype. In contrast, a second functional domain of Rev has been delineated by a series of *trans*-dominant negative mutants spanning residues 81-88 (Mermer *et al.*, 1989). These mutants retain normal RNA binding, multimerisation and localisation activities, yet are functionally negative. It has been proposed that this domain, which contains a number of critically spaced leucine residues, interacts with a cellular co-factor critical for Rev activity (Malim *et al.*, 1991). Rev therefore demonstrates a modular organisation similar to that of transcriptional activators (Ptashne, 1988; see also tat,section 1.9), i.e. a binding domain which mediates specificity, and an effector or activation domain which translates binding into a functional event. The amino-terminal 14 and carboxy-terminal 25 residues of HIV-1 Rev are dispensable for activity (Perkins *et al.*, 1989; Malim *et al.*, 1989b; Hope *et al.*, 1990).

Rev activity has also been found residing within a 28kDa phosphoprotein, Tev (Benko *et al.*, 1990). This protein contains the Tat first exon, Rev second exon and a novel coding exon

Figure 1.5: Modular Organisation of the HIV-1 Rev Protein

Domain structure of the Rev protein of HIV-1. The amino acid sequence of the RN binding and activation domains are highlighted. Region of predicted secondary structure (helix) are shown. NH2: amino terminus, COOH: carboxy-terminus. NLS: nuclear localisatio signal. The exon1/exon2 boundary is shown.

HIV-1 REV


In Vitro
Multimerisation
Domain
Exon
Boundary
1 Dispensable
23
26
Basic Domain
56
Activation Domain
116
NH2
COOH
α–helix
α– helix
Dispensable
75 LPPLERLTLD 83
35 RQARRNRRRRWRERQR 50
NLS

within Env. The apparently normal Rev activity of Tev implies that the first exon-specific residues of Rev are not essential for activity; however it is possible that the Tat/Env residues of Tev provide compensatory activity. Tev is not required for HIV-1 replication (Purcell and Martin, 1993).

The 27kDa HTLV Rex protein demonstrates a similar, though less discrete, domain structure to that of Rev (Bohnlein S. *et al*, 1991). The amino-terminal proximal 19 residues form a highly basic domain, which mediates nuclear localisation and RNA binding (Siomi *et al.*, 1988; Bogerd *et al.*, 1991). An activation domain, containing reiterated leucine residues, is present within the central region of the molecule (Hope *et al.*, 1991; Weichselbraun *et al.*, 1992).

1.11.1 THE AMINO TERMINAL DOMAIN

1.11.1.1 RNA Binding

HIV-1 Rev is a sequence specific RNA binding protein (Daly *et al.*, 1989; Zapp and Green, 1989). Mutations within the highly basic amino-terminal domain of Rev eliminate this activity (Malim *et al.*, 1989b; Olsen *et al.*, 1990; Zapp *et al.*, 1991). Synthetic peptides corresponding to this domain are able to bind target RNA in *in vitro* assays with an affinity and specificity only slightly lower that that of the full length protein (Kjems *et al.*, 1992; Tan *et al.*, 1993). Moreover, Rev specific binding characteristics can be transferred to Rex by fusion to Rev residues 33-46 (Bohnlein *et al.*, 1991), demonstrating that this region is sufficient for sequence specific binding. Scanning mutagenesis analysis of the arginine-rich (10/17 residues are arginine) binding domain has been performed in order to identify critical residues. Tan and colleagues (1993) found that substitution of individual arginine residues within a Rev peptide led to a reduction in specific binding; however, Hammerschmid *et al.* (1994) were able to replace each residue within the domain with leucine with no apparent reduction in binding. These apparently contradictory results may be explained by the fact that the latter study utilised full length Rev protein. Thus, individual mutations may be more tolerated in the context of the intact molecule, perhaps due to contributory interactions by residues outside the minimal domain. A degree of functional redundancy therefore resides within Rev. These, and other studies using protease protection analysis (Kjems *et al.*, 1992; Jensen *et al.*, 1995) have identified those residues which make specific contacts with target RNA, and those which contribute structural information to the protein. Those residues outlined in **bold** are believed to contact the RNA:

34 **T R** Q A **R R N** R R R **R** W R E R Q **R** 50

Rev belongs to a class of RNA binding molecules characterised by an arginine-rich motif (Lazinski *et al.*, 1989). This group also includes HIV-1 Tat, bacteriophage N anti-terminating proteins and non-specific RNA binding molecules such as histones. There is little sequence homology within this class beyond a preponderance of arginine residues. Reiterated arginines have a high affinity, but no specificity, for guanine-rich sequences via electrostatic interactions (Daly *et al.*, 1993a). Competition experiments suggest that Rev is essentially a poly(G) binding protein (Daly *et al.*, 1993a). Non-arginine residues within the basic domain presumably contribute discriminatory interactions as a secondary event to initial arginine-guanine contact.

Analysis of Rev structure by circular dichroism has suggested that the protein contains a high degree (40-48%) of α-helical content (Nalin *et al.*, 1990; Wingfield *et al.*, 1991). This is predicted to take the form of a helix-loop-helix motif in the amino-terminal portion of the molecule (helix 1: residues 8-26, loop 27-33, helix 2: 34-59) (Auer *et al.*, 1994). Recently, Tan and co-workers have shown that the efficiency of binding to the RRE of a binding domain peptide is directly proportional to the helical content of the peptide (Tan *et al.*, 1993). The basic domain is predicted to form a helix in both the isolated peptide and in the context of the full length molecule. The helix may make contact with the RNA within a major groove distorted by non-canonical base pairing (see section 1.12).

Multiple arginine residues also comprise the RNA binding domain of HTLV Rex proteins (Bogerd *et al.*, 1991). Substitution with lysines abrogates binding, as does alteration of the interspersed non-basic residues (Hammes and Greene, 1993). Helix destabilising proline residues within the basic domain suggest that Rex contacts RNA in a distinct manner from that of Rev (Tan and Frankel, 1995).

1.11.1.2 Subcellular Localisation

Subcellular localisation of HIV-1 and HIV-2 Rev and HTLV-I Rex proteins within the nuclei, and accumulation within the nucleoli, of transiently transfected cells is mediated by the arginine-rich domains of these proteins (Cullen *et al.*, 1988; Siomi *et al.*, 1988; Perkins *et al.*, 1989; Dillon *et al.*, 1991). Nuclear (NLS) and nucleolar (NOS) targeting signals within Rev are distinct (Cochrane *et al.*, 1990b).The SV40 T antigen NLS (sequence PKKKRKV) is the prototype of a class characterised by highly basic content and short size (Kalderon *et al.*, 1984). The Rev NLS

belongs to this group: the pentapeptide RRRRW (residues 41-45) is sufficient to direct the nuclear targeting of the normally cytoplasmic protein β-galactosidase (Perkins *et al.*, 1989). Residues flanking this peptide are required for nucleolar targeting. Whilst the entire basic domain (35-50) has been proposed to function as a minimal nucleolar localisation signal (NOS) (Miyazaki *et al.*, 1992), other groups have demonstrated that elements amino-proximal to this motif are necessary for efficient localisation (Hope *et al.*, 1990b; Venkatesh *et al.*, 1990).

The demonstration that the specific RNA binding motif coincided with the putative NLS/NOS meant that the significance of localisation is difficult to assess. However, a serine for tryptophan substitution at residue 45 abolishes nuclear localisation while preserving other functions: this mutant is inactivating, demonstrating the requirement for nuclear localisation for Rev function (Hammerschmid *et al.*, 1994). The relevance of nucleolar localisation to function has been studied by generating chimaeric Rev/heterologous RNA binding proteins and assaying for Rev activity on an RNA target containing a cognate recognition sequence for the heterologous protein. The bacteriophage MS2 RNA binding protein and its operator sequence have been used (McDonald *et al.*, 1992; Venkatesan *et al.*, 1992). This system allows Rev function to be studied independently from RNA binding. McDonald and colleagues found that a mutant which localised to the nucleoplasm but was excluded from the nucleolus possessed full function, arguing against the functional importance of nucleolar targeting.

Since nucleoli are not discrete, membrane limited organelles, accumulation implies binding to a nucleolar component. The overlap of the localisation and RNA binding domains suggests that nucleolar distribution is due to low affinity binding to ribosomal RNA (Felber *et al.*, 1989). RNase treatment of isolated nuclei abolishes Rev nucleolar binding (D'Agostino *et al.*, 1995) Alternatively, Rev may bind to a nucleolar protein. The basic domain has been implicated in high specificity binding to two acidic stretches within the abundant nucleolar protein B23 (Fankhauser *et al.*, 1991). This evolutionally conserved, 38kDa protein shuttles rapidly between the nucleus and cytoplasm, and is believed to chaperone the import of ribosomal components to the nucleolus, the site of ribosome biogenesis (Borer *et al.*, 1989). Peptides corresponding to the NOS (residues 37-47) and to the SV40 T antigen NLS, bind with high affinity and specificity to B23 in *in vitro* assays (Szebeni *et al.*, 1995). A peptide corresponding to the Rex NOS (residues 1-19) also binds B23 (Adachi *et al.*, 1993). Rev nucleolar distribution overlaps that of B23 (Luznik *et al.*, 1995). It may be that Rev/Rex can 'hijack' B23 in order to gain rapid access to the nucleus, or that B23 acts as a nucleolar receptor for Rev. Active rRNA synthesis and nucleolar

integrity are required for Rev nuclear localisation (Richard *et al.*, 1994; D'Agostino *et al.*, 1995), suggesting that Rev might be stored within the nucleolus by binding to both B23 and ribosomal RNA, until target RNA is available.

1.11.1.3 Multimerisation

A third function of the amino terminal region of Rev was characterised on the basis that mutations flanking the arginine-rich motif abrogated *in vivo* activity while preserving both localisation and *in vitro* RRE binding (Berger *et al.*, 1991). Such mutations were found to prevent Rev multimerisation on the RNA target, and spanned a bipartite domain including residues 18-31 and 51-57 (Malim and Cullen, 1991, Zapp *et al.*, 1991). Mutations outside of this domain do not interfere with *in vitro* multimerisation, as observed by the ability of Rev to form multiple, differentially migrating ribonucleoprotein complexes in gel mobility shift assays (Olsen *et al.*, 1990). Recent reports have suggested, however, that *in vivo* multimerisation may require different sequence specificities. Using an *in vivo* assay for Rev association based on Rev/Tat chimaeras, Cullen and colleagues demonstrated that the activation domain mutant RevM10 was unable to multimerise *in vivo*, despite wild type activity *in vitro* (Madore *et al.*, 1994). Mutations in the pre-defined multimerisation domain were partially active. It was concluded that *in vivo* complex formation requires stabilisation through bridging interactions with a cellular co-factor mediated by the activation domain. Similar conclusions were reached following a study of *in vivo* HTLV-I Rex association (Bogerd and Greene, 1993). Proteins with mutated leucine-rich domains were found to be deficient in multimerisation. It therefore appears that *in vitro* assays measure the intrinsic ability of Rev/Rex to multimerise on the RRE, but that such complexes are non-functional without the stabilising effect of the cellular co-factor.

1.11.2 THE ACTIVATION DOMAIN

The HIV-1 Rev activation or effector domain is a discrete, independent region of 9-10 residues (Mermer *et al.*, 1990). Mutations within this domain abolish Rev response (Benko *et al.*, 1990). All other domains of Rev are dispensable for activity if Rev is tethered to its RNA target by heterologous protein:RNA binding interactions (see 1.12): integrity of the activation domain alone is crucial (McDonald *et al.*, 1992; Venkatesan *et al.*, 1992). This domain consists of critically spaced leucine/isoleucine residues interspersed with generally hydrophilic residues of less

primary significance (Malim *et al.*, 1991). The carboxy-terminal portion of the domain displays greatest conservation both amongst HIV-1 strains and between different retroviral Rev/Rex proteins (Malim *et al.*, 1991). Leucine periodicity is a feature of several proteins involved in mediating protein:protein interactions; these include the U2 snRNP A' protein (Fresco *et al.*, 1991). This suggests that the activation domain contacts a cellular co-factor(s) essential for Rev function, a hypothesis supported by analogy with other cellular and viral *trans*-activators. Inactivating activation domain mutants display a dominant negative phenotype when co-expressed with wild type protein (Malim *et al.*, 1989b; Mermer *et al.*, 1990; Venkatesh and Chinnadurai, 1990). Such *trans*-dominant (TD) inhibition is dose-dependent and can be potent; the mutant M10 (substitution of DL for LE at positions 78,79) reduces wild type function by 20-fold when co-expressed at 10-fold molar excess (Malim *et al.*, 1989b). It has been proposed that these mutants interfere with the correct recruitment of the cellular co-factor by wild-type Rev.

Recent studies have purported to identify cellular proteins which interact with the activation domain. Early evidence derived from study of the characteristics of the activation domain made such identification possible. In particular, the activation domains of all studied Rev/Rex proteins are believed to be functionally interchangeable: this is true for those proteins with closely homologous activation domains, for example HIV-1 and SIV_{mac} Rev (Berchtold *et al.*, 1995), and for those with less distinct domain structure with limited homology to HIV-1 Rev (HTLV-I Rex) (Hope *et al.*, 1991). The Rev proteins of FIV and EIAV have larger activation domains which are characterised by polar residues rather than leucine periodicity (Mancuso *et al.*, 1994): these domains are also fully interchangeable with that of HIV-1 Rev (Fridell *et al.*, 1993; Mancuso *et al.*, 1994). All Rev/Rex proteins must therefore share a cellular co-factor, or contact different co-factors within the same cellular pathway. The conservation of the putative co-factor is demonstrated by the fact that Rev function is supported by a range of cell types, from human/primate to rodent (Olsen *et al.*, 1990), *Drosophila melanogaster* (Ivey-Hoyle and Rosenberg, 1990) and *Saccharomyces cerevisiae* (Stutz and Rosbash, 1994) cells. The co-factor is likely to be an integral part of a fundamental cellular pathway.

The potent inhibitory effect of TD Rev mutants suggests a possible role in therapeutic intervention against retroviral disease. 'Intracellular immunisation' (Baltimore, 1988) with constructs expressing TD mutants might render the cell refractory to superinfection with wild type virus: TD mutants of the regulatory protein VP16 are able to protect cells from challenge with herpes simplex virus type 1 (HSV-1) (Friedman *et al.*, 1988). Hypothetically, there are three

potential mechanisms of *trans*-dominance which may apply to Rev. These are; sequestration of a limiting co-factor ('squelching'), formation of inactive mixed multimers, and competition for target binding sites. Distinction between these possibilities is important: inhibition based on sequestration of an essential cellular factor would induce toxicity, contra-indicating such therapy.

Initial experiments favoured the mixed multimer hypothesis: TD Rev can inhibit wild-type Rev function in either the nucleus or cytoplasm (Hope *et al.*, 1992). However, subsequent demonstration that activation domain mutants were unable to support multimer formation in *in vivo* assays (section 1.11.1.2) (Daly *et al.*, 1993b; Madore *et al.*, 1994) argued against this model. Probable resolution of this question has been achieved by a series of experiments with Rev and Rex. Both of these proteins can *trans*-activate through the RRE, although they have different sites of binding (Solomin *et al.*, 1990). They do not form mixed multimers (Bogerd and Greene, 1993), although their activation domains are functionally interchangeable. A Rev TD mutant is able to inhibit Rex/RRE function (Benko *et al.*, 1990), but only in the presence of the Rev core binding site (Solomin *et al.*, 1990). On a hybrid RNA with both the RRE and RxRE, Rev TD can inhibit Rev but not Rex activity (Benko *et al.*, 1990). Inhibition cannot thus be due to formation of an inactive complex, as Rev and Rex do not multimerise. The requirement for the Rev binding domain strongly implies that Rev TD blocks Rex activity by sterically inhibiting binding to the RRE: this cannot occur through the RxRE which is not bound by Rev. The mutant cannot 'squelch' the cellular co-factor, as in this case it would inhibit both proteins on the hybrid RNA. The inability of TD mutants to support *in vivo* multimerisation further supports the inactive monomer hypothesis: TD mutants can compete efficiently for binding to the RRE, but are deficient in subsequent critical protein assembly events mediated by the co-factor.

T-Cell lines stably expressing various Rev TD mutants remain susceptible to infection but fail to support productive replication (Bevec *et al.*, 1992; Malim *et al.*, 1992). The Rev M10 mutant has been the subject of proposed somatic gene therapy against AIDS (Nabel *et al.*, 1994).

1.11.3 Phosphorylation

Rev/Rex proteins are post-translationally modified by the activity of nuclear serine kinases (Dillon *et al.*, 1991; Oberste *et al.*, 1993). HIV-1 Rev is thought to be phosphorylated at two terminal sites (Hauber *et al.*, 1988), the precise locations of which are uncertain. Serine substitution mutants, defective for phosphorylation, are active *in vivo*, demonstrating that this modification is not essential (Cochrane *et al.*, 1989b; Malim *et al.*, 1989b). In contrast, the *in vitro*

RNA target binding ability of the HTLV-II Rex protein is directly related to the degree of phosphorylation (Green *et al.*, 1992). Treatment of HTLV-I infected cells with the kinase inhibitor H-7 blocks the expression of unspliced viral mRNA, suggesting an *in vivo* role for phosphorylation state (Adachi *et al.*, 1990). Rex regulation by phosphorylation may allow the virus to monitor the physiological status of the host cell. HTLV-1 Rex is also a phosphoprotein (Kiyokawa *et al.*, 1985).

1.12 The Rev Responsive Element

Sequences acting in *cis* to confer rev responsiveness on RNA were initially described by deletion mutagenesis (Rosen *et al.*, 1988; Hadzopoulou-Cladaras *et al.*, 1989). Subsequently, these were found to coincide with a predicted highly complex region of secondary structure within the *env* RNA (Malim *et al.*, 1989c). The **r**ev **r**esponsive **e**lement (RRE: also CAR, *cis* anti-repressor) is located immediately downstream of the SU/TM encoded boundary within *env*, and thus is within the intron spliced out of the 2kb class of rev-independent mRNAs. The RRE retains activity when relocated to various positions within *env*, demonstrating its independent nature; however, it must be present in the sense orientation (Malim *et al.*, 1989c). Subsequently, several groups reported direct interaction of recombinant Rev protein with RRE-containing transcripts in *in vitro* gel mobility shift and filter binding assays (Daly *et al.*, 1989; Zapp and Green, 1989; Cochrane *et al.*, 1990a; Daefler *et al.*, 1990). Binding to the RRE is an absolute requirement for Rev activity (Dayton *et al.*, 1989; Cochrane *et al.*, 1990a). The interaction of Rev with the RRE is highly specific, with an estimated dissociation constant (Kd) of 0.8-5nM (Cook *et al.*, 1991; Heaphy *et al.*, 1990; Holland *et al.*, 1990).

Computer simulations predict that the 234nt HIV-1 RRE forms a highly stable secondary structure (ΔG_f ~450-500kJ/mol), consisting of a central stem and five stem/loops (SL$_{IIB,C-V}$) (figure 1.6) (Malim *et al.*, 1989c; Le *et al.*, 1990).The central stem (domain IA) is formed by base pairing between complimentary nucleotides at the 5' and 3' termini of the RRE. Deletion mutagenesis and RNase protection assays were performed to both confirm the predicted RRE secondary structure, and to delineate the specific elements required for Rev binding and function. Recent evidence suggests that an extended stem$_I$ is required to mediate full activity; this defines a 351nt element (Kimura and Ohyama, 1994; Mann *et al.*, 1994). An 88nt fragment encompassing SL$_{IIB}$ and stem$_{IIA}$ and a portion of Stem$_{IA}$ is able to confer full

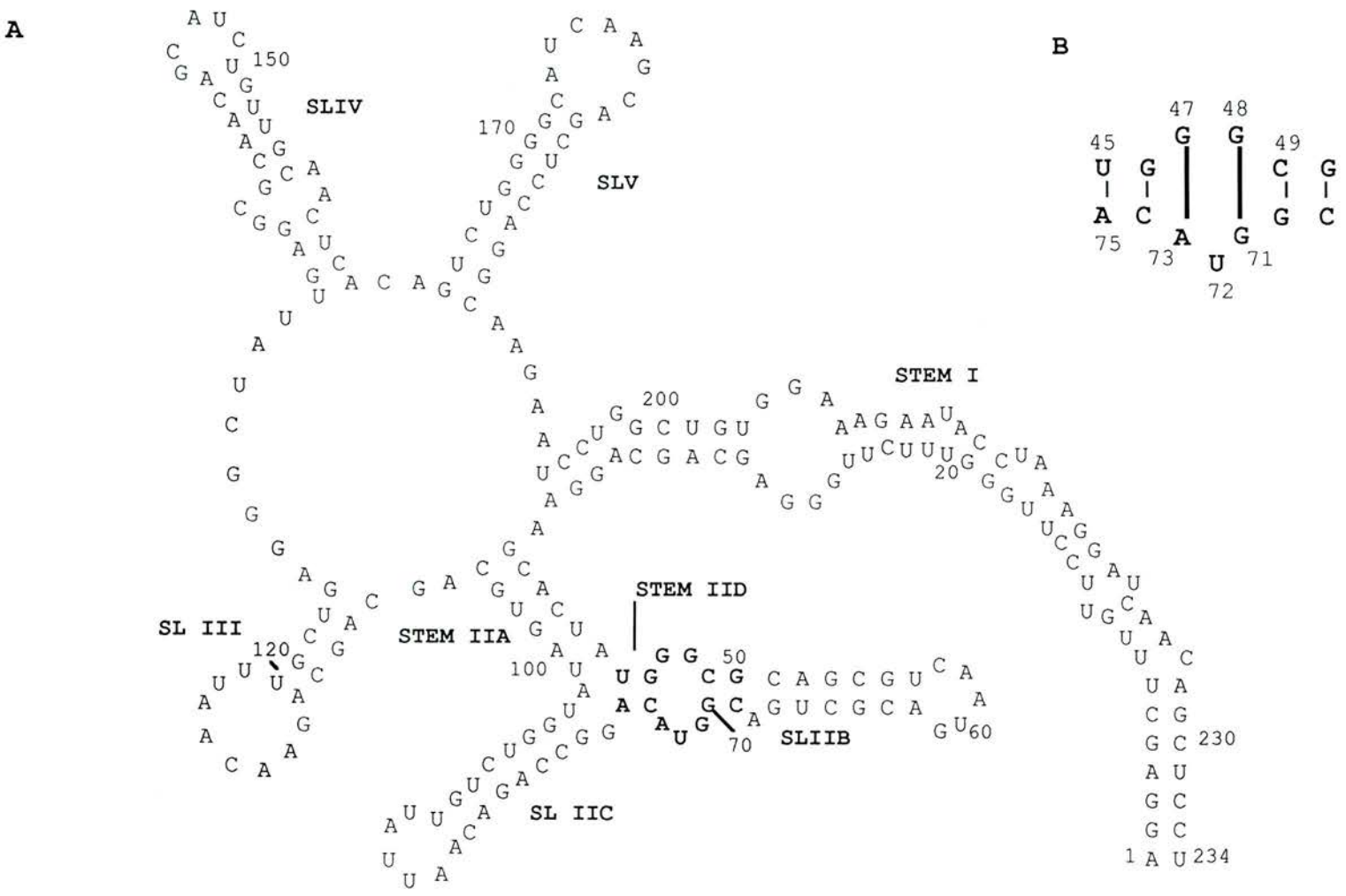
Figure 1.6: The HIV-1 Rev Responsive Element (RRE)

A

Computer prediction of the secondary structure of the Rev responsive element from th
HXB-3 strain of HIV-1 (based on Malim *et al.*, 1989c, Bartel *et al.*, 1991). Nucleotide positions a
given in reference to the RRE, where position 1 is equivalent to genomic location 7348. Th
experimentally elucidated core Rev binding element is highlighted in **bold**. SL: stem loop.

B

The minimal Rev binding element (RBE). Putative non-canonical (purine-purin
interactions are depicted.


A
B
SLIV
SLV
STEM I
STEM IID
STEM IIA
SL III
SLIIB
SL IIC
150
170
200
120
100
50
70
60
20
230
234
1
75
73
72
71
47
48
49
45

responsiveness when present as a dimer in rev-dependent sub-genomic constructs (Huang *et al.*, 1991). Further dissection of this region led to the identification of the base of SL IIB as the specific, core Rev binding site (Dayton *et al.*, 1989; Holland *et al.*, 1990; Malim *et al.*, 1990). This high affinity **R**ev **b**inding **e**lement (RBE) consists of ~13 non-contiguous nucleotides (figure 1.6b) (Heaphy *et al.*,1991). Although the experimentally defined location of the RBE was supported by all authors, there was initial disagreement concerning both the contribution to Rev recognition of sequence-specific versus structure specific-information, and of the base pairing arrangements within the RBE. The interdependence of nucleotides with respect to overall secondary structure and the limited knowledge of protein:RNA interactions in general may have contributed to this. Bartel and colleagues (1991) presented evidence favouring a structural recognition motif. In this model, the RBE consists of an asymmetric internal loop surrounded by helical duplex RNA. The internal loop, or bulge, is purine rich and is characterised by non Watson-Crick base pairing between nucleotides G48 and G71, and between G47 and A73. Significantly, although substitution of a single A nucleotide for either G48 or G71 abrogates *in vitro* Rev binding, a double substitution with A results in full activity. Homopurine base pairing at this position would be expected to distort the RNA sugar-phosphate backbone, resulting in sufficient widening of the narrow major groove for protein access. Without this distortion, the groove is likely to be too deep and narrow for protein entry (Weeks and Crothers, 1993).

Recent evidence has both supported and extended the latter model. Attempts to synthesise high affinity, synthetic Rev ligands by iterative selection of a random pool of RNA sequence variants have generated oligomers which resemble the Bartel model (Giver *et al.*, 1993; Jensen *et al.*, 1994). In particular, these studies have confirmed the importance of isosteric G:G or A:A base pairing at RRE positions 48 and 71. Ligands with homopurine pairs bind Rev with up to 160x greater affinity than other pairings. Chemical protection (Tiley *et al.*, 1992), nucleotide analogue (Iwai *et al.*, 1992) and NMR studies (Battiste *et al.*, 1994; Peterson *et al.*, 1994) have also proved valuable in discerning features of the Rev-RNA interaction. Certain nucleotides are invariable and are likely to make specific contacts with Rev. The affinity of Rev for the minimal RBE is less than that for the entire RRE, suggesting that non-specific contacts with flanking bases are important *in vivo* (Tiley *et al.*, 1992). Other nucleotides make a structural contribution to the recognition motif. Thus, for example the looped out residue U72 cannot be deleted from the RBE; however, it can be replaced with any other residue with no loss of function. This suggests that it acts as a spacer to accommodate distortions of the helix

introduced by the flanking non-canonical base pairing. Rev binding may induce conformational changes in the RRE, including stabilisation of the purine bulge (Kjems *et al.*, 1991a; Battiste *et al.*, 1994).

The HIV-1 protein, Tev, which displays Rev activity, is competent to bind to the RRE. However, it possesses a relaxed binding specificity (Solomin *et al.*, 1990). This is probably a result of the presence of the Tat basic motif within the amino-terminal region of Tev (Benko *et al.*, 1990).

The minimal RBE is insufficient to provide full Rev responsiveness *in vivo*. Vectors incorporating the entire SLII sub-domain support approximately 50% of wild-type response (Holland *et al.*, 1992). The presence of a portion of stemI duplex RNA can increase this to 80%, probably by providing low affinity binding sites for full Rev multimerisation. Rev binds to the minimal RBE with one to one stoichiometry (Cook *et al.*, 1991). The high affinity binding event induces the recruitment of additional Rev molecules to the RNA by co-operative oligomerisation along SLIIB, Stem IIA and Stem I. Scatchard analysis of Rev oligomers suggests that secondary binding involves low affinity protein:RNA interactions (Heaphy *et al.*, 1991), and is primarily driven by protein:protein contacts. Rev monomers will bind to a low affinity site on a nucleated RNA in preference to a free, high affinity site (Mann *et al.*, 1994). Co-operative binding also results in stabilisation of the Rev:RNA interaction: the half life for RNA association of a Rev molecule at the RBE increase from 7-9 minutes to 20-25 minutes on binding of a second monomer (Daly *et al.*, 1993a). Visualisation of oligomeric complexes by gel retardation electrophoresis has demonstrated that up to eight monomers can bind to a single transcript (Daly *et al.*, 1989; Heaphy *et al.*, 1991; Wingfield *et al.*, 1991). At saturating Rev concentrations and appropriate conditions, Rev will form a filamentous structure up to 1.5mm in length on both RRE and heterologous duplex RNAs (Heaphy *et al.*, 1990).

The question of whether Rev binding is the only role of the RRE has been addressed by tethering Rev to target RNA via a heterologous protein-RNA interaction. Near wild type levels of trans-activation can be achieved if SLII is exchanged for the bacteriophage MS2 operator element or HIV-1 TAR if Rev is fused to the cognate binding protein (Luo *et al.*, 1993; McDonald *et al.*, 1992; Venkatesan *et al.*, 1992). However, exchange of the entire RRE reduced responsiveness by 50-65%. These experiments highlight two points: firstly, that at least a substantial proportion of the response does not depend on any unique features of the RRE beyond its Rev binding characteristics, and, secondly, that RRE domains outside of the binding

regions contribute a quantitative, facilitating role to Rev responsiveness. Cellular factors which reportedly bind the RRE are therefore unlikely to have a significant role in the Rev response. These factors include a 56kDa ubiquitous nuclear protein (Vaishnav *et al.*, 1991) and two human proteins, p62 and p120 (Shukla *et al.*, 1994). Deletion of the additional, non binding domains (SLIIC, III, IV and V) results in a small, though distinct, loss of responsiveness to Rev, supporting the conclusions of the heterologous RNA-binding experiments. For example, a ΔSLIV or V mutant retains ~90% wild type function; however a double deletion mutant retains only ~35% function (Holland *et al.*, 1990; Malim *et al.*, 1990). A degree of functional redundancy therefore exists within the RRE. These domains are not binding sites; they are susceptible to nuclease digestion even in the presence of saturating Rev concentrations (Mann *et al.*, 1994). The primary sequence of domains outwith the binding elements is generally not critical (Dayton *et al.*, 1992). It is possible that they stabilise the overall structure of the RRE, and may ensure that the binding elements are presented in an appropriate structural context for Rev binding.

The Rex response elements (RxRE) of HTLV-I is situated within the U3/R region of the viral LTR. It forms a predicted complex secondary structure of 254nt composed of two stems and four stem loops (SLA-D) (Hanly *et al.*, 1989). High affinity and specificity binding of Rex to *in vitro* synthesised RxRE has been demonstrated (Bogerd *et al.*, 1991; Unge *et al.*, 1991): binding correlates with function *in vivo* (Ballaun *et al.*, 1991). The high affinity minimal binding site for Rex has been isolated. It forms a stem:bulge:stem structure of ~12 nt within the central stem of SLD (Bogerd *et al.*, 1992; Grone *et al.*., 1994). Rex is also able to bind and function through the HIV-1 RRE (Bogerd *et al.*, 1991), though with different specificity to Rev, requiring stem loops III, IV and V (Solomin *et al.*, 1990). This is probably the result of the fortuitous presence of a suitable binding element, and suggests that Rex makes less discriminatory contacts than Rev. The core RxRE binding site is well conserved between HTLV-I and HTLV-II and STLV (Bogerd *et al.*, 1992). HTLV-II Rex binds to this site on the HTLV-II RxRE (Kim *et al.*, 1991).

The RxRE is predominantly located within the R region of the LTR. Elements in U3 are not absolutely required for activity (Kim *et al.*, 1991). Formation of the 3' RxRE juxtaposes the widely spaced polyadenylation signal and cleavage site, the 3' RxRE is therefore present within all transcripts (Ahmed *et al.*, 1991). Thus unspliced, genomic RNA contains two copies of the element. The major splice donor site is located within the 5' RxRE; utilisation of this site removes the core binding region. Spliced transcripts thus contain a single 3' RxRE. The basis of the selective action of Rex on the incompletely spliced structural mRNA in these viruses is not clear:

negative regulatory elements may exist in the coding regions of these transcripts to differentiate them from multiply spliced, Rex independent mRNA.

1.13 THE MECHANISM OF REV FUNCTION

1.13.1 Retroviral Regulatory Mechanisms

Once integrated, the retroviral provirus is effectively a cellular gene. Transcription by the host cell RNA polymerase II generates a pre-mRNA primary transcript. The retrovirus primary transcript serves a three-fold function: as genomic RNA, as mRNA for gag and pol, and as pre-mRNA for further processing to produce mRNA for env and the auxiliary genes if these are present. The retrovirus thus has to overcome two problems: functional splice sites are required within the primary transcript but a proportion of unspliced transcript must be retained, and this transcript must be exported to the cytoplasm for translation and/or virion assembly, despite incomplete processing.

The so-called 'simple' retroviruses employ *cis*-acting regulatory elements within their genomes to regulate viral RNA processing and overcome these problems. Gene expression in these viruses is constitutive. The 'complex' retroviruses employ an additional regulatory strategy, the Rev/RRE axis, which allows temporal control of viral RNA processing and the consequent biphasic pattern of viral replication. In order to reach an understanding of the mechanisms of retroviral regulation, it is necessary to review the current understanding of the fate of RNA from transcription to translation in eukaryotic cells.

1.13.2 mRNA Processing

Extensive processing of eukaryotic pre-mRNAs is required to produce mature mRNAs competent for translation. Transcripts undergo 5' cap formation, methylation, 3' end cleavage, polyadenylation and splicing to remove intervening sequences (introns). Processing is thought to follow a highly integrated pathway, culminating in RNA translocation to the cytoplasm. Nascent pre-mRNAs are co-transcriptionally coated with heterogeneous nuclear ribonucleoproteins (hnRNPs) (reviewed by Laskey and Dingwall, 1993). The composition of the RNP may determine the fate of the nascent RNA.

Introns are present in the majority of cellular genes; for many splicing is a requirement for

efficient processing and nucleocytoplasmic export. Incompletely spliced RNAs are generally retained within the nucleus. Retention is followed by further splicing or degradation (Ryu and Mertz, 1989). Splicing is catalysed by a multi-subunit RNP complex, the spliceosome. Assembly of this complex occurs as a step-wise process, with rapid initiation on nascent transcripts (reviewed in Lamond, 1993). A large number of **s**mall **n**uclear **r**ibon**u**cleo**p**roteins (snRNPs), RNAs (snRNAs) and other splicing factors are involved. Many of these make specific contacts with the substrate pre-mRNA at various points, including the 5' and 3' splice sites. An early step in spliceosome assembly is the formation of a 'commitment complex' which may mark the RNA for splicing (Seraphin and Rosbash, 1989). It was thought that binding of U1 snRNP to the 5' splice site was responsible for formation of the commitment complex (Legrain *et al.*, 1988; Seraphin and Rosbash, 1989). Recent research, however, points to the involvement of a class of non-snRNP splicing factors, the SR (serine-arginine rich) proteins (Fu, 1993). Since splicing and nucleocytoplasmic translocation of most cellular transcripts are tightly coordinated, it has been proposed that the commitment complex acts to retain pre-mRNA within the nucleus. In yeast, certain splice site mutations result in increased cytoplasmic expression of pre-mRNA (Legrain and Rosbash, 1989) suggesting that transport of pre-mRNA can occur if spliceosome assembly is blocked.

The nucleocytoplasmic transport of most RNAs is dependent on prior processing events (reviewed in Izaurralde and Mattaj, 1995). Splicing is a general prerequisite for export, and disruption of this leads to nuclear retention. Kinetic data suggests that RNA translocation is an energy dependent, saturable process, indicating that it is signal mediated. Furthermore, different classes of RNA follow distinct export pathways (Jarmolowski *et al.*, 1994). This points to the involvement of specific *trans*-acting factors possessing appropriate export signals which mediate ligand translocation.

1.13.3 Viral *Cis*-Regulation

Certain retrovirus splice sites do not conform to the cellular consensus sequences. This results in inefficient association with the splicing machinery and creates a pool of unspliced viral RNA (Katz and Skalka, 1990). Creation of consensus splice sites by mutation results in attenuated replication in simple retroviruses, such as Rous sarcoma virus (RSV) and avian sarcoma virus (ASV) (Katz *et al.*, 1988; Zhang and Stoltzfus, 1995). The RSV genome also contains a number of discrete *cis*-regulatory elements which regulate splicing through binding of

cellular splicing factors. For example, an intragenic *src* suppressor of splicing (SSS) site inhibits utilisation of the downstream splice acceptor site by interaction with an avian nuclear factor (McNally and Beemon, 1992; Amendt *et al.*, 1995). HIV-1 also contains suboptimal splice sites and *cis*-regulatory elements which regulate the complex mRNA balance (Amendt *et al.*, 1994; Staffa and Cochrane, 1995).

Thus a pool of incompletely spliced transcripts is generated by the action of *cis*-regulatory elements. Fusion of such elements to heterologous genes shows that unspliced RNA is retained within the nucleus (Arrigo and Beemon, 1988). Nucleocytoplasmic transport of this RNA occurs constitutively in cells infected with simple retroviruses. A recently discovered 219nt element within the 3' untranslated region (UTR) of the type D retrovirus Mason-Pfizer monkey virus (MPMV) appears to mediate translocation, and has been named the constitutive transport element (CTE) (Bray *et al.*, 1994). Intronless genes are unlikely to access the mRNA splicing/export pathway, and have been hypothesised to contain *cis*-acting signals to specify transport (Buchman and Berg, 1988). The intronless hepatitis B virus contains a post-transcriptional regulatory element (PRE) which can activate the cytoplasmic accumulation of heterologous unspliced RNA when present in *cis* (Huang and Liang, 1993; Huang and Yen, 1995). The 119nt pre-mRNA processing enhancer (PPE) element is located within the intronless thymidine kinase gene of herpes simplex virus type-1. Binding of the PPE RNA to hnRNP L correlates with cytoplasmic accumulation (Liu and Mertz, 1995). Although it is unclear by what mechanism hnRNP L mediates this effect, it is likely that the MPMV and HBV elements contact this or similar factors.

1.14 Viral *Trans*-Regulation

1.14.1 The Rev Phenotype

Although the role of Rev in facilitating the expression of incompletely spliced viral RNAs is clear, the mechanism by which this is achieved is not. Some uncertainty concerning the distribution of viral RNA species with respect to Rev function also remains. In part, the use of diverse reporter constructs and cell types to assay Rev function may explain this. Much of the work performed to evaluate the role of Rev has involved transient transfection of transformed cell lines using sub-genomic viral fragments in a non-viral vector context. Data from the study of the other auxiliary gene products suggests that the function of lentiviral proteins may vary in different

cell types. Many studies have utilised sub-genomic HIV constructs transfected into COS cells. It is well established that the Rev deficient phenotype is associated with a high ratio of multiply spliced to incompletely spliced viral RNA in the cytoplasm. The increase in incompletely spliced RNA in the cytoplasm on transfection with a Rev expression plasmid is coincident with a reduction in the level of multiply spliced transcripts. Thus, in these cells, Rev exerts negative feedback control over its own production (Felber *et al.*, 1990). Nuclear events are less clear. It has been reported that incompletely spliced transcripts are present regardless of Rev activity such that Rev functions to equilibrate the cytoplasm with a pool of pre-existing unspliced RNA. As these transcripts are the substrate for splicing, the level of multiply spliced RNAs will fall. Alternatively, some reports suggest that the total cellular level of unspliced RNAs is very low in the absence of Rev, due to splicing and/or instability. Felber and co-workers found that the half life of incompletely spliced RNA increased from under one hour to greater than six on Rev co-expression (1989).

A recent report by Malim and Cullen (1993) is significant: these authors used a Rev attenuated provirus established by authentic retroviral infection of T lymphocytes. It was found that in these cells, Rev has no quantitative effect on the multiply spliced RNAs. Instead, Rev activity induced a six-fold increase in the total amount of unspliced, 9kb RNA, due to enhanced stability and concomitant nucleocytoplasmic translocation. It was concluded that the nucleus harbours two pools of primary viral transcript; one that is a substrate for splicing and one that is retained and degraded, and that Rev acted on this latter pool only. The physical difference between these pools is unexplained; however differences in RNA fate in heterologous cell types may be a consequence of varied kinetics of RNA processing events. For example, degradation of unspliced transcript may occur at a faster rate in T-lymphocytes compared to COS cells.

1.14.2 Determinants of Rev Dependence

As discussed above, RNA which is dependent on Rev for expression is defective in the absence of Rev. This is a result of nuclear retention (Rosen *et al.*, 1988) degradation (Felber *et al.*, 1989) or inefficient translation (Arrigo and Chen, 1991) in the absence of Rev. It is presumed that *cis*-acting signals mediate these fates, and hence specify Rev dependence. It is unclear whether splice sites are required to specify Rev dependence, or whether intragenic *cis*-acting repressive sequences (CRS) possess this property.

1.14.2.1 Role of Splice Sites

Inefficiently utilised splice sites have been shown to be required for Rev dependence. Replacement of authentic sites with efficient β-globin splice sites in a Rev dependent construct results in rapid splicing and Rev independent expression (Chang and Sharp, 1989). Inhibition of splicing by mutation of either splice site led to nuclear retention of the intron-containing transcript, presumably a result of formation of a commitment complex unable to progress to functional assembly. This was overcome by Rev/RRE interaction. Mutation of both splice sites to prevent commitment complex formation resulted in constitutive nucleocytoplasmic export (Chang and Sharp, 1989). Thus it was proposed that Rev dependence required the presence of sub-optimal splicing signals retaining the ability to interact with the spliceosome machinery and specifying nuclear retention. Rev may function by resolution of such snRNP complexes, allowing export by a default mechanism. Other researchers found that a residual 5' splice site was both necessary and sufficient to determine Rev dependence: binding of this site to U1 snRNA was implicated in nuclear retention (Lu *et al.*, 1990). Hammarskjold *et al.* (1994) extended these observations, finding that *env* must be defined as an intron for Rev dependence. These results are highly controversial. Many authors have proposed that Rev dependence is retained in the absence of utilisable splice sites (for example Emerman *et al.*, 1989; Malim *et al.*, 1989c). It is possible that these transcripts retain the ability to form commitment complexes, even if they are unable to progress to splicing. Elimination, as opposed to mutation, of all splice sites has also been shown to preserve Rev dependence (Nasioulas *et al.*, 1994): however, deletion of known splice sites can reveal cryptic ones (Wieringa *et al.*, 1983). Recent work in yeast cells has suggested that partial entry of target RNA into a splicing commitment complex is necessary to potentiate Rev function (Stutz and Rosbash, 1994).

1.14.2.2 CRS Elements

Elements other than splice sites have been proposed as the determinants of Rev dependence. Several intragenic inhibitory elements have been identified which repress expression of viral genes independently of the presence of functional splice sites. Two groups have identified such elements which function in constructs where all viral splicing information has been deleted (Brighty and Rosenberg, 1994; Nasioulas *et al.*, 1994). These elements are known as *cis*-acting repressive sequences (CRS) (Rosen *et al.*, 1988). The effect of the CRS elements

is overcome by deployment of the Rev/RRE regulatory axis. Regions with CRS activity have been identified within the *gag*, *pol* and *env* genes of HIV-1 and HIV-2, and within HIV-2 *vif* (see table 1.2). The LTR R/U5 region of HTLV-I and-II contain similar activities (Seiki *et al.*, 1990; Black *et al.*, 1991). There is no evident homology between these sequences, some of which probably overlap. Several of these elements have been shown to reduce the expression of chimaeric heterologous genes (Rosen *et al.*, 1988; Dayton *et al.*, 1988; Cochrane *et al.*, 1991; Schwartz *et al.*, 1992a), suggesting that they can function as independent units. Multiple CRSs may have an additive inhibitory effect (Maldarelli *et al.*, 1991). It is particularly intriguing that a CRS within *env* may overlap the RRE (Nasioulas *et al.*, 1994; Brighty and Rosenberg, 1994). A reduction in expression of heterologous reporter genes on insertion of the RRE has been reported by several groups (Huang *et al.*, 1991; Holland *et al.*, 1992), although this has not been universally observed (Chang and Sharp, 1989). Brighty and Rosenberg have suggested that this CRS may suffice to ensure Rev-dependence of a transcript. A simplistic model of Rev function would have Rev competing for a sterically limited binding site with factor(s) which mediate reduced expression via the CRS.

CRS elements may introduce defects into viral RNA at the level of instability, nuclear sequestration and/or inefficient utilisation, which prevent functional expression. Several groups have demonstrated the nuclear retention of CRS-containing RNA (see table 1.2). In contrast, a *gag* CRS identified by Schwartz and co-workers (1992a) acts as an instability element which reduces the steady state level of RNA in all cellular compartments. This element (INS-1) is characterised by a high AU content (61.5%). Similar AU-rich elements (AURE) are present in the 3' untranslated regions of several cellular genes which are subject to rapid up- and downregulation, for example *c-fos* and *interleukin 1* (reviewed in Ross, 1995). Insertion of *gag* INS-1 into the HIV-1 *tat* mRNA reduces its half life from four hours to under one (Schwartz *et al.*, 1992a). AUREs are often characterised by the pentamer AUUUA, although this sequence does not feature in INS-1. However, there are several copies of this sequence within the structural genes of HIV-1 and other lentiviruses (Schwartz *et al.*, 1992b). INS-1 can be inactivated by site-directed mutagenesis to lower the AU content: this results in Rev-independent expression of *gag* (Schwartz *et al.*, 1992b).

Table 1.2: *Cis*-Acting Repressor Sequences

VIRUS	GENE	LOCATION	MECHANISM	REFERENCE
HIV-1	GAG	287-1549	Not stability	Had.-Clad. 89
	GAG	414-631	Instability	Schwartz 92
	GAG	711-2006	Nuclear retention	Maldarelli 91
	POL	2619-4551	Nuclear retention	Maldarelli 91
	POL	3795-4055	Translational ?	Cochrane 91
	ENV	6004-7595	NI	Nasioulas 94
	ENV	6376-6725	NI	Rosen 88
	ENV	7382-7623	Nuclear retention	B & R 94
	ENV	7735-7989	Nuclear retention	Emerman 89
HIV-2	VIF	5020-5160	Instability?	Reddy 95
	ENV	8570-8760	Nuclear retention	Keller 93
HTLV-I	LTR	450-700	NI	Seiki 90
HTLV-II	LTR	520-630	Nuclear retention	Black 91

Genomic location and proposed mechanism of inhibition of described *cis*-acting repressive sequences (CRS) elements within the genomes of HIV-1, HIV-2, HTLV-I and HTLV-II. It is likely that some of these elements overlap. NI: not investigated. Had.-Clad.: Hadzopoulou-Cladaras *et al.*, 1989. B & R: Brighty and Rosenberg, 1994.

In addition to the effect on stability, AUREs can inhibit translation (Kruys *et al.*, 1989). The precise mechanism of CRS inhibition may be context dependent. The *pol* CRS identified by Cochrane *et al.* (1991) mediates nuclear retention in an HIV background, but reduces translation efficiency of a CRS/CAT chimaera.

CRS inhibition is probably mediated by the binding of cellular proteins. Several AU-binding proteins have been characterised (for a review see Decker and Parker, 1995, and references therein). Two cellular factors which bind the HTLV-II LTR CRS have been isolated (Black *et al.*, 1994). These proteins have recently been identified as two members of the hnRNP family, hnRNP A1 and polypyrimidine tract binding protein (or hnRNP1) (Black *et al.*, 1995). Binding of hnRNP A1 was found to be necessary for CRS mediated inhibition of gene expression. Olsen and colleagues (1992) have identified a 42kDa nuclear factor, a putative

member of the hnRNP C family, which binds to a CRS within *pol*. hnRNP C is a splicing factor which binds to intronic sequences (Bandziulis *et al*., 1989). The CRS may elicit the formation of ribonucleoprotein complexes refractory to nucleocytoplasmic export. It is possible that these complexes also protect pre-mRNA from splicing, thus preserving a pool of incompletely spliced transcripts available for the late stage of viral replication.

In summary, several, distinct CRS elements downregulate the expression of lentiviral structural genes by a variety of mechanisms. The significance of these elements with respect to the determination of Rev dependence is still to be conclusively established. The recent work of Brighty and Rosenberg (1994) strongly supports the role of CRSs. These authors found that *env*-derived constructs which lacked the RRE associated CRS but possessed known splice sites were expressed independently of Rev; constructs with the opposite content were Rev dependent. It is also possible that there is functional redundancy within the complex retroviral genome, with both splice sites and CRS elements interacting with nuclear factors to retain a pool of incompletely spliced viral RNA.

1.15 Rev Functional Mechanisms

The mechanism of Rev function has not been fully characterised. A direct effect of Rev on splicing would superficially explain the observed Rev phenotype. Rev cannot be a general inhibitor of splicing, however, as it promotes the expression of the singly-spliced 4kb class of transcripts concurrently with the unspliced genomic transcripts. A second proposed model for Rev function is that Rev chaperones target RNAs from the nucleus to the cytoplasm. Cytoplasmic activities have also been proposed for Rev.

1.15.1 Direct Inhibition of Splicing

A nuclear factor detected in HIV-1 infected cells was found to inhibit the splicing of pre-mRNAs in *in vitro* assays (Gutman and Goldenberg, 1988). Subsequent analysis by Kjems and colleagues has suggested that the basic domain of Rev may mediate this phenomenon. Using an *in vitro* assay based on splicing of a construct containing an RRE within the intron and splice sites derived from the β-globin pre-mRNA, this group identified a 3-4 fold reduction in splicing due to the full length Rev protein (Kjems *et al*., 1991b). A more significant 30-fold inhibitory effect was achieved by use of a synthetic peptide corresponding to Rev residues 34-50. Further

study purported to identify the mechanism of Rev inhibition: prevention of entry of the U4/U6.U5 tri-snRNP complex into the maturing spliceosome (Kjems and Sharp, 1993). Since the basic domain was entirely responsible for this effect, it was proposed that conformational changes in the RNA induced by binding to the RRE resulted in spliceosome assembly arrest. A proposed interaction between the basic domain and the murine protein YL2, a homologue of human p32, which co-purifies with the splicing factor SF2/ASF (Krainer *et al.*, 1991) lent support to this model (Luo *et al.*, 1994). SF2/ASF has been implicated in formation of a specific spliceosome commitment complex involving the HIV-1 tat pre-mRNA (Krainer *et al.*, 1990): sequestration of this factor by Rev could reduce splicing. The relevance of these observations is questioned by the finding that HIV-1 Tat basic domain also binds p32 (Fridell *et al.*, 1995). More significantly, the role of the basic domain has been shown to be limited to discriminatory RNA binding by the binding of Rev chimaeras to heterologous targets (McDonald *et al.*, 1992; Venkatesan *et al.*, 1992). Indeed, other observations strongly suggest that Rev has no direct *in vivo* anti-splicing activity. Rev has no quantitative or qualitative effect on the distribution of multiply spliced mRNAs in a T lymphocyte cell line (Malim and Cullen, 1993), despite a dramatic effect on unspliced RNA: Rev action in these natural viral host cells therefore cannot involve inhibition of splicing. Furthermore, by using *Xenopus* oocytes, Rev activity has been shown to occur in cells simultaneously with splicing of transcripts containing the RRE (Fischer *et al.*, 1994): Rev function is therefore independent of any inhibitory effect on splicing.

Thus, despite some circumstantial evidence to the contrary, HIV-1 Rev does not appear to have a role in the direct inhibition of splicing. However, Rev/Rex proteins of other viruses may have such an activity. In both the HTLVs and EIAV, Rev/Rex binding to the response element has been speculated to inhibit splicing at neighbouring splice sites, possibly by steric hindrance. Rev activity correlates with a reduction in the production of a four exon multiply spliced EIAV mRNA due to alternative splicing to remove the third exon (Martarano *et al.*, 1994). In the HTLVs, the splice donor site utilised to produce *env* and *tax/rex* mRNA is situated within stem-loop D of the 5' RxREs, close to the core Rex binding element. In HTLV-II, Rex activity requires the presence of this site (Black *et al.*, 1991). Although suggestive of a role for Rex in steric inhibition of splicing, there is no experimental evidence to support this hypothesis.

1.15.2 Nucleocytoplasmic Export

Early reports suggested that Rev could increase the efflux of RRE-RNA from isolated

vesicles (Pfeifer *et al.*, 1991). Investigation into this model for Rev activity has been hampered by the rudimentary state of current knowledge of the mechanisms of RNA export. However, recent reports suggest that Rev function may become a paradigm for specific export mechanisms.

As noted above (1.13.2) RNA may be escorted through the nuclear pore complex by specific transport factors. Several proteins which 'shuttle' between the nuclear and cytoplasmic distribution have been linked to the cytoplasmic accumulation of distinct classes of RNA. For example,hnRNP A1 may mediate the export of poly(A)$^+$ mRNA (Piñol-Roma and Dreyfuss, 1992), and TFIIIA has been linked to the export of 5S rRNA (Guddat *et al.*, 1990). Mutations which prevent 5S rRNA from binding to TFIIIA result in RNA nuclear retention.

The recent demonstration that HIV-1 Rev is a shuttle protein (Kalland *et al.*, 1994; Meyer and Malim, 1994; Richard *et al.*, 1994) strongly suggests that it is a viral equivalent of such factors, directly involved in mediating export of its ligand RNA. The originally described nuclear/nucleolar localisation of Rev masks a dynamic situation. Transfection of cells expressing Rev with a reporter construct expressing RRE-RNA induces the rapid cytoplasmic localisation of the Rev protein (Richard *et al.*, 1994; Luznik *et al.*, 1995). However, the presence of RRE-RNA is not essential for shuttling. Inhibition of nuclear import of Rev by disruption of RNA polymerase activity with actinomycin D or 5,6-dichlorobenzimidazole riboside (DRB) resulted in Rev accumulation in the cytoplasm (Meyer and Malim, 1994; D'Agostino *et al.*, 1995; Szilvay *et al.*, 1995), regardless of the presence or absence of RRE-RNA. However, Rev molecules with mutations of the activation domain remained in the nucleus under these conditions. The biological activity of a series of activation domain mutants was found to correlate exactly with the ability to enter the cytoplasm (Meyer and Malim, 1994). This is compelling evidence that the primary biological action of Rev is to directly facilitate the nucleocytoplasmic export of target mRNAs.

The activation domain is thus a putative nuclear export signal sequence (NES). Confirmation of this has come from an elegant series of experiments recently reported by Fischer *et al.* (1995). Intranuclear injection of BSA conjugated to synthetic peptides resulted in rapid cytoplasmic accumulation if the peptide corresponded to the wild-type activation domain (AD) of Rev or Rex, but to nuclear entrapment if a non-functional mutant (M10) was used. Identical results were achieved by Wen *et al.* (1995), who also identified a leucine-rich NES in the cellular protein kinase inhibitor (PKI). BSA-AD competitively inhibited both Rev function and the nuclear egress of 5S rRNA and of certain snRNAs, though not that of mRNA, tRNA or other ribosomal

components (Fischer *et al.*, 1995). This suggests that this export signal is recognised by a single, saturable cellular factor, responsible for translocation. TFIIIA, implicated in 5S rRNA export, contains a homologous leucine-rich motif: this protein may be a cellular Rev/Rex homologue (Fischer *et al.*, 1995). These export signals are compared below: interspersed hydrophobic, generally leucine, residues (**bold**) are the major defining characteristic.

HIV-1 Rev	75	**L**	P	P	**L**	E	R	**L**	T	**L** 83
HTLV-I Rex	84	A	Q	**L**	**Y**	S	S	**L**	S	**L** 92
PKI	39	**L**	K	-	**L**	A	G	**L**	D	**I** 46
TFIIIA	58	**L**	S	T	**V**	A	**V**	**L**	T	**L** 66

A putative factor has recently been isolated from both human (Bogerd *et al.*, 1995; Fritz *et al.*, 1995) and *Saccharomyces cerevisiae* (Stutz *et al.*, 1995) cells. These proteins bind specifically to wild type, though not to inactivating mutant, activation domains of Rev and Rex. The human variant, RIP (**r**ev **i**nteracting **p**rotein) (Fritz *et al.*, 1995) or RAB (**r**ev/**r**ex **a**ctivation domain **b**inding protein) (Bogerd *et al.*, 1995), is a 58-59kDa protein with sequence features characteristic of human nucleoporins, protein components of the nuclear pore complex. These include a high serine/threonine content, and numerous phenylalanine/glycine repeats. Rip/Rab appears to localise predominantly in the nucleoplasm of transfected cells, with some distribution in both the nucleolus and cytoplasm. This is not the expected distribution of a nucleoporin, suggesting that Rip/Rab may function to transfer Rev/Rex from the nucleolus/nucleoplasm to the perinuclear region, where it may 'dock' with the pore complex. Both groups found that overexpression of Rip/Rab significantly enhanced Rev function. Bogerd and co-workers demonstrated that Rip/Rab possessed several genetically pre-determined characteristics of the Rev co-factor. These included a perfect correlation between activation domain mutant binding and function, distribution in a range of cell types able to support Rev function, binding to Rev as part of a Rev/RRE-RNA complex, and the ability to bind heterologous activation domains, such as that of EIAV Rev, which can functionally replace HIV-1 Rev activation domain (Fridell *et al.*, 1993; Bogerd *et al.*, 1995). The yeast variant, **R**ev **i**nteracting **p**rotein-**1 p** (RIP1p) (Stutz *et al.*, 1995) has a similar composition. Yeast mutants not expressing RIP1p do not support Rev function, but maintain viability. Rip1p may thus be a member of a class of functionally redundant molecules. Rip1p differs from its human homologue in its distribution at nuclear pores. Moreover,

overexpression of Rip1p dampens Rev response in yeast. This discordant finding may be due to different levels of each factor in yeast and human cells, such that lower levels in human cells are supplemented by overexpression, but higher background levels in yeast cells lead to 'squelching' of Rev on overexpression.

Thus, Rev may function as a 'molecular bridge' connecting RRE containing RNAs with a specific section of the nucleocytoplasmic export machinery. Investigation of the effect on viral replication in natural target cells of ablation of Rip/Rab expression is an obvious future experiment. If Rip/Rab is not essential for cell viability in humans, then ablation of expression is a possible strategy for anti-HIV therapeutic intervention.

1.15.3 Translational Regulation

A number of researchers have recorded a potential discrepancy in the upregulatory effect of Rev on structural protein synthesis as opposed to the increase in cognate mRNA (Sodroski *et al.*, 1986; Hadzopoulou-Cladaras *et al.*, 1989; Benko *et al.*, 1990; Chanda *et al.*, 1990; D'Agostino *et al.*, 1992). These findings have been taken as evidence for a role for Rev in translational regulation (D'Agostino *et al.*, 1992). However, these data often refer to Rev-induced increases in total cellular mRNA, which may not reflect the differential increase in cytoplasmic mRNA occasioned by Rev action. However, the universality of Rev-induced alterations in mRNA distribution has been challenged by other authors. Arrigo and Chen (1991) identified singly spliced vif, vpr and env/vpu 2 mRNAs in the cytoplasm of Rev deficient lymphoid cells (729 B cells). Rev function had little effect on the distribution of these mRNAs. Conventional results were recorded in COS cells. Gag mRNA has been observed in the cytoplasm of HeLa cells expressing ΔRev proviruses (D'Agostino *et al.*, 1992). In both these systems, mRNA found in the cytoplasm in the absence of Rev was translated inefficiently. This defect was found to be due to exclusion of RNA from polysomes. Instead, RNA was associated with a 40-80S ribonucleoprotein complex (Arrigo and Chen, 1991), which may be an arrested pre-initiation complex. Incompletely spliced mRNAs expressed in the cytoplasm of lymphoid cells in the absence of Rev were found to lack association with poly(A) binding protein (PAB1) (Campbell *et al.*, 1994). PAB1 is necessary for the completion of polysome formation (Sachs and Davis, 1989); thus the failure of mRNA to interact with PAB1 would be expected to prevent efficient translation. Pavlakis and co-workers proposed that CRS-mediated defects in RNA conformation or RNP

composition might prevent poly(A) association, and that Rev would function to correct this (Campbell *et al.*, 1994). *In situ* hybridisation experiments have revealed that Rev remains bound to RNA molecules within the cytoplasm of infected cells (Arrigo *et al.*, 1992), where it may allow functional association with the translational machinery. Support for the translational regulatory role of Rev comes from the identification of eukaryotic translation initiation factor 5A (eIF-5A) as a putative Rev co-factor (Ruhl *et al.*, 1993). EIF-5A is a 19kDa hypusine residue containing protein with an unknown *in vivo* role (for a review of initiation factor biology, see Park *et al.*, 1993). *In vitro*, this protein catalyses the formation of the dipeptide analogue methionyl-puromycin, mimicking the formation of the first peptide bond during translation (Benne and Hershey, 1978), although this activity might be artefactual (Kang *et al.*, 1993). Rev may recruit eIF-5A to improve the efficiency of translation of bound RNAs, perhaps by mediating the correct association of PAB1. Since eIF-5A has both nuclear and cytoplasmic distribution (Ruhl *et al.*, 1993) it may have a further role in Rev activity.

EIF-5A was isolated as a Rev activation domain binding factor. Evidence for the functional importance of eIF-5A is three-fold. Firstly, inhibition of eIF-5A expression with antisense oligonucleotides blocked Rev function (Ruhl *et al.*, 1993). Secondly, Rev activity was not observed in *Xenopus laevis* oocytes: exogenous eIF-5A supported Rev function. Finally, exogenous eIF-5A was found to reconstitute Rex function in a system where the Rev/Rex co-factor was sequestered by a *trans*-dominant inhibitory mutant (Katahira *et al.*, 1995). Several objections to this evidence may be raised. Fischer *et al.* (1994) found Rev to be fully functional in *X. laevis* oocytes, directly contradicting Ruhl and colleagues. Although Ruhl *et al.* demonstrated binding of an activation domain synthetic peptide to eIF-5A, they did not characterise binding to the full-length protein, nor demonstrate lack of binding to known activation domain mutants. It should be noted that others have failed to repeat the results of Ruhl *et al.* (Bogerd *et al.*, 1995). Moreover, although it has been genetically determined that Rev and Rex have a common co-factor (Hope *et al.*, 1991), Katahira and co-workers were unable to significantly reconstitute Rev function with eIF-5A.

The significance of the data concerning an effect of Rev on translation, and its possible interaction with eIF-5A is uncertain. In particular, most investigators have not observed large amounts of Rev dependent mRNA in cytoplasmic fractions in the absence of Rev. Although there may be cell type specific effects, Malim and Cullen (1993) were unable to repeat the findings of Arrigo and Chen in CEM lymphoid cells. The latter authors employed quantitative PCR

analysis to assess the nature of cytoplasmic RNAs. This technique would be sensitive to any leakage of material from nuclear extracts during cell fractionation, which may explain the discordant results. However, it remains a possibility that a proportion of RNAs may escape nuclear retention by an unproductive route or as nonfunctional RNP complexes in certain cell types. Rev would be required to redirect RNA through productive translocation and on to efficient polysomal association.

1.15.4 A Unified Theory for Rev Activity

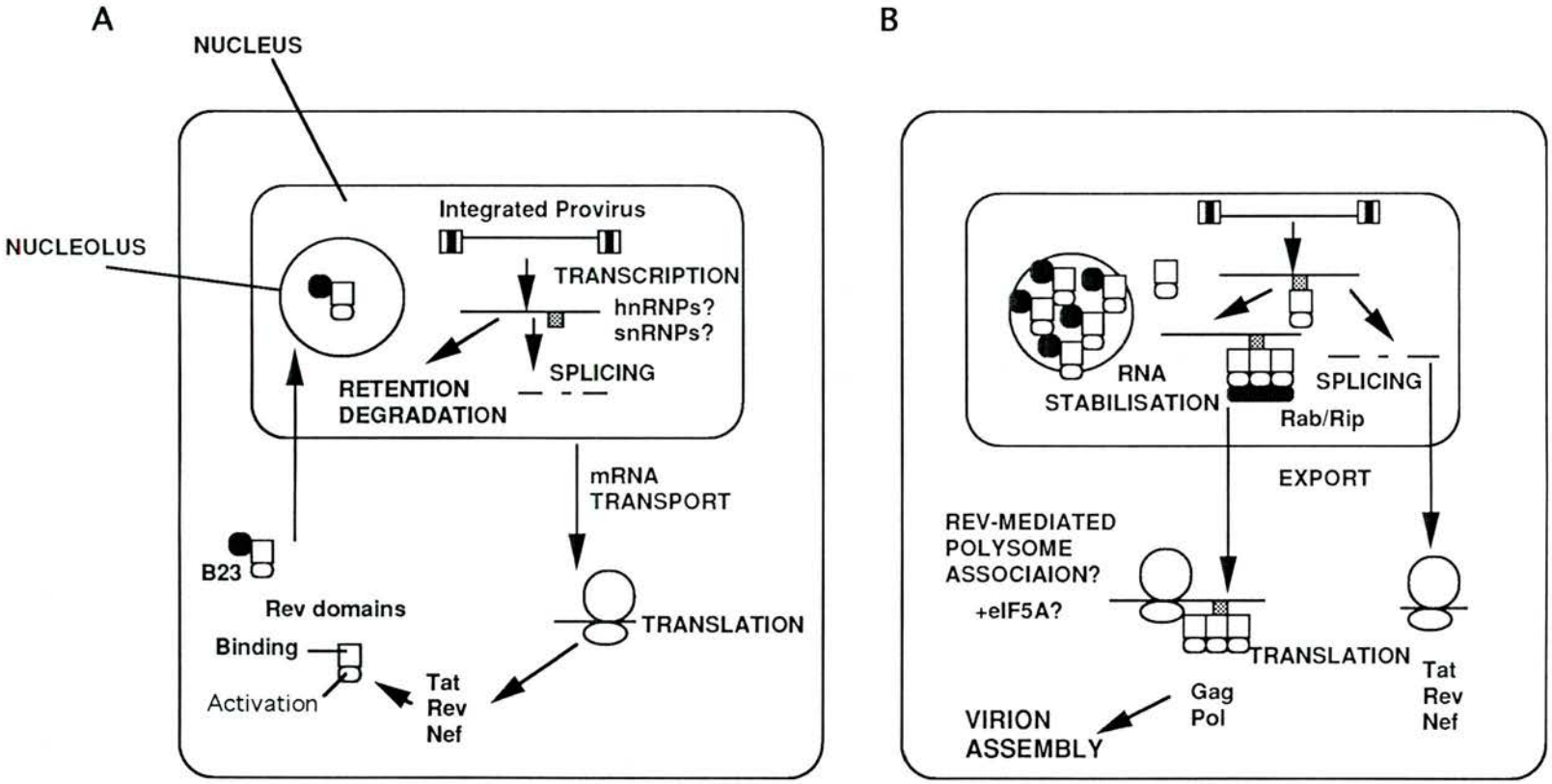
A possible model of HIV-1 Rev function based on the data currently available follows, and is illustrated by figure 1.7.

The integrated HIV-1 provirus remains quiescent until host cell activation or differentiation induces transcription from the viral LTR. The early phase of transcription produces the regulatory proteins Tat, Rev and Nef. Tat initiates a positive feedback loop, *trans*-activating viral expression. Competitive interaction of cellular nuclear factors with various viral *cis*-acting signals determines the fate of the viral primary transcript. The primary transcript is either spliced to completion, or retained in the nucleus. Degradation may occur. Rev protein produced from multiply spliced RNAs is localised to the nucleus via the nuclear localisation signal: this event may be mediated by interaction with the shuttle protein B23. Once within the nucleus, Rev may be retained within the nucleolus by binding to B23 or to ribosomal RNA. Rev monomers may bind to their core RNA target within the RRE, present on viral primary transcripts. However, this interaction is insufficient to affect the fate of these transcripts. Once a critical intranuclear concentration of Rev has been reached, rapid multimerisation, driven by highly co-operative binding events, along the duplex RNA within the RRE occurs. The reiterated Rev activation domains mediate interaction with a co-factor, Rip/Rab. This event stabilises the Rev oligomer. Nucleocytoplasmic transport of the Rev/RNA complex then ensues, probably mediated by 'docking' of the co-factor with the nuclear pore complex. The transcript is translated, although interaction with a second co-factor, eIF-5A, may be required for functional association with the translational machinery. Rev protein released from the transcript may return to the nucleus to initiate further RNA translocation. Expression of the structural gene products marks the late phase of replication, with packaging of the RNA genome and virion assembly.

Thus the primary mode of action of Rev may be to mediate nucleocytoplasmic transport

Figure 1.7: Possible Model for the Activity of HIV-1 Rev

A proposed unified hypothesis for the function of HIV-1 Rev. Early (A) and late
phases of viral replication are discernible. For simplicity, the fate of singly spliced viral RNA h
been excluded. For discussion, see text.


A
NUCLEUS
NUCLEOLUS
Integrated Provirus
TRANSCRIPTION
hnRNPs?
snRNPs?
SPLICING
RETENTION
DEGRADATION
mRNA
TRANSPORT
B23
Rev domains
Binding
Activation
Tat
Rev
Nef
TRANSLATION
B
RNA
STABILISATION
SPLICING
Rab/Rip
EXPORT
REV-MEDIATED
POLYSOME
ASSOCIAION?
+eIF5A?
TRANSLATION
Tat
Rev
Nef
Gag
Pol
VIRION
ASSEMBLY

of RNA containing the RRE. By acting as a chaperone for RNA, Rev activity might by-pass divergent RNA processing and regulatory mechanisms. The presence and kinetics of such mechanisms might vary between cells, accounting for the reported variation in Rev attenuation phenotypes. Thus, the Rev/RRE interaction is sufficient to overcome the inhibitory effect of heterologous *cis*-acting repressive elements. An AURE in the 3' untranslated region of the structural gene mRNAs of human papillomavirus types 1 and 16 reduces both the half life and translatability of these transcripts (Kennedy *et al.*, 1991; Tan and Schwartz, 1995). This effect is counter-acted by Rev and the RRE (Tan and Schwartz, 1995). Rev also increases the expression of hybrid transferrin receptor/RRE mRNAs which are otherwise rapidly degraded (Zolotukhin *et al.*, 1994). It must be concluded that the action of Rev in 'rescuing' defective RNA is a general one.

Confirmation of the validity of the above model is awaited. In particular, it will be necessary to demonstrate physical association of the putative co-factor with the Rev/RNA ribonucleoprotein complex, and with the nuclear pore complex.

1.15.5 NS1, An Orthomyxovirus Rev Homologue?

Influenza A virus is an orthomyxovirus with a genome consisting of eight RNA segments. Two of these, 7 and 8, express additional mRNAs via splicing events (Lamb and Lai, 1980; Inglis and Brown, 1981). Segment seven encodes non-structural protein 1 (NS1) from the unspliced RNA, and NS2 from a spliced derivative. Thus, a similar problem to that faced by retroviruses exists. The balance of splice products is controlled by both *cis*-acting signals, and by a *trans*-acting factor, NS1 (Alonso-Caplen and Krug, 1991). NS1 consists of 237 amino acid residues and has a modular construction strikingly similar to Rev/Rex. An amino-terminal domain, which mediates binding to poly(A) RNA, a carboxy-terminal effector domain and two nuclear localisation signals are discernible (Qian *et al.*, 1994; Qiu and Krug, 1994). Although the effector domain consists of ~30 residues, it is characterised by the presence of critical periodically spaced leucine residues. Moreover, multimerisation is required for activity (Nemeroff *et al.*, 1995). It might therefore be expected that NS1 is functionally analogous to Rev. However, NS1 mediates the inhibition of nucleocytoplasmic export of target RNAs (Alonso-Caplen *et al.*, 1992). It is possible that NS1 contacts the same co-factor as Rev, and that the additional effector domain residues are required to inhibit rather than facilitate transport. Alternatively, a distinct co-factor requirement

exists. NS1 has been reported to possess a plethora of activities, including splicing inhibition and stimulation of translation of viral transcripts (De La Luna *et al.*, 1995). How these activities are co-ordinated to regulate viral replication is unknown.

1.16 Rev and the Molecular Basis of Latency

A Δ*rev* HIV-1 provirus constitutively expressed in cell culture can be rescued to complete replication by transfection with a Rev expression vector. By titrating the Rev vector, Pomerantz and co-workers demonstrated that the relationship between induced virus replication and Rev expression was non-linear (1992). A critical threshold level of Rev expression was required to substantially increase virus production. The threshold effect may be advantageous to the virus: it acts as a 'dam' allowing high level expression of viral RNA in the absence of corresponding levels of structural proteins sensitive to immune surveillance: once the critical level has been reached, the accumulated RNA can initiate high level production of structural proteins to optimise the production of progeny virions.

The molecular basis of the threshold effect is thought to be the requirement for Rev multimerisation on target RNA for activity (Malim and Cullen, 1991; Heaphy *et al.*, 1992). Below the threshold concentration, Rev would bind as a monomer to the high affinity site on the RRE. At the critical concentration, oligomerisation of Rev nucleated by the high affinity binding event would be induced. This would occur rapidly, due to the highly co-operative nature of Rev multimer assembly. Optimal Rev activity is associated with full multimerisation along the RRE SL$_{IIB}$ and Stem $_{IA}$ (Mann *et al.*, 1994). Indeed, Mann and co-workers have described the RRE as a 'molecular rheostat', detecting and responding to the intracellular Rev concentration (1994).

As has been discussed, lentivirus persistence in the host is, at least partially, a result of restricted viral expression. Theoretically, maintenance of a sub-threshold Rev concentration would be one method to restrict virus expression. Certain chronically infected cell lines have been proposed as models of *in vivo* HIV latency. Transcriptional suppression underlies the restricted viral state in two of these cell lines, the T lymphocyte-derived ACH-2 and monocyte-derived U1 (Folks *et al.*, 1989). However, a sub-threshold Rev concentration acts as a proximal block to expression, preventing structural protein synthesis from the low basal level of viral transcripts which are maintained in an aberrant pattern ('blocked-early') similar to that seen in the early phase of single cycle productive infection (Pomerantz *et al.*, 1990). Transduction with a

retroviral vector expressing Rev induces a small increase in structural protein synthesis (Duan *et al.*, 1994). A blocked-early transcript pattern is also demonstrated by the astrocytoma cell line TH4-7-5 (Kleinschmidt *et al.*, 1994). However, the restricted state of virus expression cannot be increased by mitogenic stimulation, or by co-expression of exogenous Rev (Neumann *et al.*, 1995). Moreover, a heterologous *gag* construct cannot be regulated by Rev in these cells, strongly suggesting that a cellular block to Rev activity exists. Since fusion with rev-permissive cells does not relieve this block, it is probable that astrocytes contain factors antagonistic to Rev activity. Low Rev activity is directly responsible for restriction in these cells. Virus can be rescued from these cells by co-cultivation with monocytes, suggesting that latently infected astrocytes may be a reservoir for viral spread within the central nervous system.

Study of viral RNA patterns in chronically infected cell lines may have relevance for natural infection. Qualitative and quantitative analysis has shown that viral RNA in PBMCs may be a surrogate marker for disease progression. Deterioration in $CD4^+$ cell count is associated with increase in total viral RNA levels. Several groups have studied viral RNA patterns in longitudinal surveys of HIV^+ individuals using quantitative reverse transcriptase-initiated PCR. These have found that a shift in the predominant expression of multiply spliced RNAs to that of unspliced RNAs correlates with disease progression (Seshamma *et al.*, 1992; Furtado *et al.*, 1995; Michael *et al.*, 1995b). A stable ratio of unspliced to multiply spliced transcripts is associated with a more benign clinical course. It should be noted that this pattern has not been universally observed (Saksela *et al.*, 1994). Thus, in asymptomatic individuals the predominant viral expression pattern is similar to the blocked early stage latency seen in chronically infected cell lines, and associated with a lack of rev activity. This may be the indirect result of reduced transcription, as in ACH-2 and U1 cells. However, rev attenuation as the direct cause of restriction cannot be discounted. Approximately 10% of HIV-1 infected individuals are classified as slow progressors, or long term survivors (Sheppard *et al.*, 1993). Iversen and co-workers have found that long term survivors may host attenuated viral variants, the presence of which correlates with, and contributes to, the failure of disease progression (1995). In particular, an inactivating leucine to isoleucine substitution at position 78 within the Rev activation domain was found to persist for over 4.5 years in one individual. Reversal of this mutation partially restored replication competence in cloned isolates. All long term survivors studied possessed a proportion of putative attenuating mutations within the *rev* gene. It is possible that *rev* attenuation is selected for in order to maintain

the restricted state. Eventual immune system collapse removes this selective pressure in most individuals; however others may avoid this fate due either to characteristics of their immune response or to the nature or degree of virus attenuation.

1.17 The Rev/RRE Axis of Heterologous Complex Retroviruses

A rev ORF is identifiable in all lentiviral genomes. A similar phenotype is universally associated with lack of functional rev expression (Cheng *et al.*, 1990; Kalinski *et al.*, 1991; Phillips *et al.*, 1992; Oberste *et al.*, 1993; Martarano *et al.*, 1994). However, a degree of divergence does exist. The rev/RRE axes of non-primate lentiviruses have yet to be fully characterised. Non-primate lentiviral *rev* genes share a characteristic exon location, with the first coding exon coincident with, and in the same reading frame as, the amino-terminal portion of *env* (Mazarin *et al.*, 1988; Saltarelli *et al.*, 1990; Stephens *et al.*, 1990; Kiyomasu *et al.*, 1991; Oberste *et al.*, 1993). Consequently, the signal sequence of non-primate lentiviral Env proteins is located at a more distal location within Env than is the case in other retroviruses (Sonigo *et al.*, 1985). The non-primate lentiviruses are also characterised by large size relative to the primate proteins, encoding 120-186 residues compared to 86-116 residues within the primate lentiviral group. These details support the possible classification of the primate and non-primate lentiviruses as separate evolutionary clades (1.2), and may point to the presence of additional functional capabilities within these proteins.

Limited overall sequence identity exists between the various Rev proteins (figure 1.8a); the only significant homologies being amongst the primate viruses. However, the two functional domains elucidated in HIV-1 Rev are generally conserved (figure 1.8b and c). The functional reciprocity of the putative activation domains has been discussed (1.11.2). Hydrophobic residues at positions 9 and 11 seem to have greatest significance. A basic motif can be observed in all proteins. However, those of both BIV and EIAV Rev are less distinct, with a lower proportion of basic residues. The overall modular organisation of EIAV, BIV and FIV Rev appears to be less distinct that that of the other proteins. An experimental definition of the RNA binding domains of these proteins is awaited with interest. Both HIV-2 and the various SIVs possess homologous RREs to HIV-1 in similar genomic locations (Le *et al.*, 1990). Specific binding of the Rev proteins from these viruses to cognate RREs *in vitro* has been reported (Garrett and Cullen, 1992). Both FIV and EIAV demonstrate considerable divergence with respect to RRE structure and location.

Figure 1.8: Sequence Comparisons between Rev/Rex Proteins

A

Overall amino acid sequence identity between selected complex retrovirus Rev/F proteins. Pairwise comparisons were made using the 'BESTFIT' program of the University Wisconsin Genetics Computing Group package (version 8). Percentage identities were obtair by division of the total number of identical aligned residues by the number of residues in smaller protein. Shown in **bold** are those comparisons giving greater than 30% amino a identity.

Sequences used for comparison (with reference for *rev* gene sequence) were following: HTLV-$1_{C91/PL}$ (Rimsky *et al.*, 1988), HIV-1_{HXB3} (Malim *et al.*, 1988), HIV-2_{ROD} (Gar and Cullen, 1992), SIV_{mac239} (Regier and Desrosiers, 1990), $SIV_{agmSA21}$ (Sakai *et al.*, 19 $SIV_{mndMD12}$ (Sakai *et al.*, 1991), MVV_{ev1} (Sargan *et al.*, 1991), $CAEV_{CO}$ (Saltarelli *et al.*, 19 BIV_{106} (Oberste *et al.*, 1993), FIV_{Pet} (Phillips *et al.*, 1992) and $EIAV_{Wyo}$ (Stephens *et al.*, 199

B

Comparison of the experimentally defined (HTLV-1, HIV-1, HIV-2, SIVs) or puta (remainder) RNA binding domains of the Rev/Rex proteins of the viruses listed above (A). protein sequences from the Maedi Visna virus variants 1514 (Sonigo *et al.*, 1985) and SA-OM (Querat *et al.*, 1990) are also included for comparison. Alignments made using the UWG program, 'PILEUP'.

C

Comparison of the experimentally defined (HTLV-1, HIV-1, HIV-2, SIVs, EIAV, FIV putative (remainder) activation domains of the Rev/Rex proteins of the viruses listed above Hydrophobic residues are marked in *italics*. The numbers 1,3,6,9,11 refer to residue positi within the domain, with respect to the leucine residue at HIV-1 Rev position 73. A pa alignment of the EIAV activation domain can be made, however the entire functional impor domain is shown below.

A

	HIV1	HIV2	MAC	AGM	MND	MVV	CAEV	BIV	FIV	EIAV
HTLV-1	18	20	18.7	17.1	22.9	15.5	15	16.1	13.7	10.9
HIV-1		38	31.8	31.7	24	17.2	18.1	24.1	13.8	21.6
HIV-2			57	40.2	25	18	17	26	22	26
SIVmac				40.2	37.8	18.7	19.6	27.1	23.4	15.9
SIVagm					29.3	17.1	13.4	22	17.1	28
SIVmnd						26	16.7	27.1	18.8	24
MVVev1							24.2	18.2	17.6	17.6
CAEV								25	16.7	20
BIV									20.9	18.2
FIV										18.3

B

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MVVev1    71 ...KTRRRKG WFKWLRRLKA R 88
SA-OMVV   74 ...KTKRRKG WFQWLRKLRA R 91
MVV1514   73 ...KTKKKRG WYKWLRKLRA R 90
HIV-2     33 ASQRRNRRRR WKQRWRQ... . 49
SIVmac    33 ANQRRQRKRR WRRRWQQ... . 49
HIV-1     35 RQARRNRRRR WRERQR.... . 50
SIVagm    28 ....ARQRRR ARRRWRQQQD . 43
SIVmnd    43 ....ARQRRR RKQQLRTRRA . 58
HTLVrex    1 ...MPKTRRR PRRSQRKR.. . 15
FIV       82 KMKKKRQRRR RKKKAFKK.. . 99
BIV       93 .....QNRRK QERRLSGLDR R108
EIAV      71 ....QHTPSR RDRWIRGQ.. . 84
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C

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                1   3     6     9   11
HIV-1      73 L Q L P P L E R L T L D 84
HIV-2      71 Q T I Q H L Q G L T I Q 82
MVVev1    102 S N V A G L E K L T L E 113
MVV1514   104 S N M V G M E N L T L E 115
SA-OMVV   105 G N C A G L E N L T L G 116
CAEV       91 P C L G A L A E L T L E 92
SIVmac     71 L A I Q Q L Q N L A I E 82
SIVmnd     72 L D R G P D Q L C Q G V 83
SIVagm     62 R L A D E A Q H L A I Q 73
BIV       145 G H L P P R S Y F K L K 156
HTLV1REX   82 L S A Q L Y S S L S L D 93
EIAV       41 W C R V L R Q S L P E E 52

FIV        95 KAFKKMMTDLEDRFRKLFGSPSKDEYT 120
EIAV       32 PQGPLESDQWCRVLRQSLPEEKIP  55
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In FIV, the RRE forms an extended single stem loop and is positioned at the 3' end of *env* (Phillips *et al.*, 1992). EIAV may encode multiple RREs. Interestingly, these elements are not predicted to contain a high degree of secondary structure, implying that EIAV Rev utilises a novel mechanism for RNA recognition (Martarano *et al.*, 1994). This correlates with the diverse basic motif of EIAV Rev.

Determination of the functional reciprocity of Rev/RRE interactions between the various lentiviral systems has provided important insights into Rev activity and delineation of functional domains. Moreover, it may provide an insight into the phylogenetic relationship between these viruses. Restricted reciprocity is likely to be a factor of RNA binding specificity. The relatively relaxed binding specificity of Tat may explain the complete interchangeability of all primate lentiviral Tat proteins (Sakai *et al.*, 1993). HTLV-1 Rex binds the HIV-1 RRE, but the reverse is not true (Solomin *et al.*, 1990), thus providing a molecular basis for the observed incomplete functional interchangeability (Rimsky *et al.*, 1988). However, the inability of HIV-2 and SIV_{mac} Rev proteins to function through the HIV-1 RRE (Lewis *et al.*, 1990; Sakai *et al.*, 1991) is a result of a failure to multimerise on the heterologous target (Garrett and Cullen, 1992). This suggests that the HIV-2/SIV proteins make more discriminatory contacts than the promiscuous HIV-1 Rev and HTLV-1 Rex molecules.

The recent discovery of a *rev*-like ORF within the genome of an endogenous human retrovirus, HTDV (human teratocarcinoma derived virus), is intriguing (Lower *et al.*, 1995). This virus is thought to have been extant for approximately 30 million years. The presence of this ORF suggests strong selective pressure in favour of the Rev/RRE regulatory system, and that this system may have arisen by selection on at least two occasions. Alternatively, the presence of conserved binding and activation domains between HIV-1, HTLV-1 and HTDV may implicate a more recent recombination event as the source of these genes.

1.18 Maedi Visna Virus Rev: Aims of Research Project

Data concerning the putative *rev* gene of MVV available at the onset of this research project was limited and somewhat contradictory. A MVV homologue to the HIV-1 *rev* gene was hypothesised by Vigne and co-workers (1987), who demonstrated early and late phase viral expression patterns. They suggested that the 1.2kb multiply spliced mRNA, expressed in the early phase, might encode this gene. The location and splicing pattern of this mRNA was also

suggestive of HIV *rev* (Sargan and Bennet, 1989). Subsequently, this mRNA was shown to give rise to a 19-21.5 kDa protein in *in vitro* translation systems (Mazarin *et al.*, 1988; Davis and Clements, 1989). This product reacted with immune sera from MVV infected sheep (Davis and Clements, 1989; Mazarin *et al.*, 1990), indicating expression *in vivo*. Initially termed VEP1 (viral early protein 1), the protein was renamed Rev on the basis of limited sequence similarity with the Rev proteins of HIV-1, HIV-2 and SIV (Gourdou *et al.*, 1989).

The multiply spliced, 1.2-1.4kb mRNA containing *rev* specific sequences may include three or four exons (see figure 1.3) (Mazarin *et al.*, 1988; Gourdou *et al.*, 1989): these forms differ in the presence or absence of the small, non-coding second exon. The first coding exon of *rev* is derived from the amino terminal region of *env*: these genes share an initiation codon. The predicted signal peptide of MVV Env is located between residues 78-100 (Sonigo *et al.*, 1985); hence cleavage of this peptide will result in the absence of common Rev/Env residues in the mature Env glycoprotein. The second coding exon overlaps the 3' region of *env* but is translated in a different reading frame. The 1514 MVV isolate encodes a protein of 167/168 residues, depending on the viral variant (Mazarin *et al.*, 1988; Davis and Clements, 1989). Although the overall sequence identity with other Rev proteins is low, the highly basic and leucine-rich domains are intact (figure 1.8).

Analysis of the putative Rev protein by indirect immunofluorescence and protein labelling in virally infected cells identified a cytoplasmic distribution, with a degree of association with the membranous component after cell fractionation (Mazarin *et al*, 1990). This is in direct contrast to the nuclear localisation of other Rev/Rex proteins (Cullen *et al.*, 1988; Siomi *et al.*, 1988). A peptide motif with homology to a domain of HIV/SIV Nef is present in the carboxy-terminal region of the Rev protein of the 1514 MVV strain used for these studies. As Nef demonstrates cytoplasmic/membrane localisation (see 1.8.2) this homology might have significance. However, although the common motif was implicated as functionally important in Nef (Guy *et al.*, 1990), this conclusion has been challenged (Kaminchik *et al.*, 1990).

A second unexpected finding was the ability of Rev to *trans*-activate transcription from the viral LTR (Mazarin *et al.*, 1988). A later study failed to confirm such activity (Davis and Clements, 1989).

Thus, the function and activities of MVV Rev were uncertain at this point. Although the possession of a domain structure similar to that of the more characterised Rev proteins is strongly suggestive of a shared biology, sequences unique to MVV Rev could mediate novel regulatory

mechanisms. The divergence of HIV and MVV Tat proteins demonstrate that proteins which mediate similar effects may do so by different mechanisms.

It was decided to investigate the nature of the MVV Rev protein, in an attempt to establish the mode of function. A *rev* gene was cloned from EV-1, a variant of MVV isolated from the peripheral blood mononuclear cells of a British sheep displaying symptoms of maedi. Computer prediction, based on the nucleotide sequence of EV-1, indicated the presence of a highly structured RNA element within the *env* gene. In order to examine the possible interaction between the Rev protein and this element, it was decided to perform a series of *in vitro* binding assays. This would require quantities of recombinant Rev protein in a non-denatured state, and *in vitro* transcribed RNA corresponding to the predicted response element. If interaction could be established *in vitro*, the significance of this in a cellular context would be established by assays based on transient transfection with reporter constructs. Finally, the effect of the observed variation between the *rev* gene sequences and predicted RREs of MVV isolates would be examined by reciprocal functional studies.

CHAPTER TWO

MATERIALS AND METHODS

MATERIALS AND METHODS

The materials used during research for this thesis were obtained from the following sources, unless otherwise stated. Chemicals were supplied by Sigma Chemical Company, Poole, Dorset. Tissue culture and bacteriological media were supplied by Gibco/BRL, Paisley, Strathclyde. Radiochemicals were purchased from Amersham International, Amersham, Buckinghamshire. DNA modifying enzymes, including restriction endonucleases, were obtained from several sources: Boehringer Mannheim, Lewes, East Sussex; New England Biolabs., Hitchin, Hertfordshire; Northumberland Biologicals Ltd., Cramlington, Northumberland; Promega Ltd, Southampton, Hampshire. Organic solvents were obtained from BDH Laboratory Supplies, Poole, Dorset. Methods are described as most commonly used. Modifications to the basic methods are noted in the main text.

2.1 Bacterial Culture

2.1.1 Bacterial Strains

The genotypes of the strains of *E. coli* used are detailed in Table 2.1

TABLE 2.1

GENOTYPES OF *E. COLI* STRAINS

Strain	Genotype	Reference
JM101	[F', *tra*D36, *pro*AB$^+$, *lacI*^{q}ZΔM15] *sup*E, *thi*, Δ(*lac-pro*AB)	Yanisch-Perron *et al*, 1985
DH5α	F-, Φ80d *lacZ*ΔM15, *rec*A1, *end*A1, *gyr*A96 *thi*-1, *hsd*R17 (r_k^-,m_k^+), *sup*E44, *rel*A1, *deo*R, Δ(lacZYA-*arg*F)U169	Gibco/BRL
Y1090	F', Δ*lac*U169, *pro*A$^+$, Δ(lon), *ara*D139, strA, *sup*F [*trp*C22::Tn10(tet^r)], *hsd*R(r_k^-,m_k^+) (pMC9,amp^r)	Huynh *et al*, 1985
BL21	F$^-$, *hsd*S(r_b^-,m_b^-), *gal*, *omp*T, Δ(lon)	Grodberg and Dunn, 1988

Strain JM101 was used for transformation of plasmids for routine subcloning. For large scale purification of plasmid DNA strain DH5α was used. These strains were also used for the expression of recombinant protein using the pGEX system (2.12). Two protease deficient strains, BL21 and Y1090, were also used for this purpose. As the pGEX system involves selection of transformed cells on the basis of ampicillin resistance, Y1090 cells which had lost the pMC9 plasmid, which includes an ampicillin resistance gene, were required. These were obtained by picking satellites to major colonies grown on agar plates containing ampicillin.

2.1.2 Bacterial Media and Culture Conditions

The standard medium for bacterial growth was Luria Bertani (LB) broth. This consisted of 1% w/v tryptone, 0.5% w/v yeast extract, 0.171M NaCl, pH 7.5. All plasmid vectors used contained the ampicillin resistance gene as a selective marker; hence, selective media was prepared by supplementation of LB with 125μg/ml ampicillin (LB/amp). LB/amp plates were made by the addition of 1.5% w/v bacteriological agar. Addition of 200mg/ml 5-bromo-4-chloro-3-indolyl β–galactopyranoside (X-gal) to LB/amp plates allowed for blue/white colour selection of cells transformed with the pCR™II plasmid from the TA cloning kit (section 2.7.1). Unless otherwise stated, incubation of bacterial cultures was performed at 37°C with vigorous shaking.

2.1.3 Bacterial Transformation

2.1.3.1 Preparation of Competent Bacteria

A modification of the method of Hanahan (1983) was used to produce bacterial cells competent for transformation.

A single colony of the appropriate strain of *E. coli* was inoculated into 5ml of LB. This preculture was grown to saturation and 200μl diluted into 20ml of an enriched medium, psi broth. After incubation to an OD_{550} of approximately 0.3, 5ml of this culture was diluted into 100ml psi broth and grown to an OD_{550} of 0.48. After brief cooling on ice, the cells were recovered by centrifugation at 2500-3000g for 5 minutes at 4°C. Pellets were resuspended in 33ml of ice cold transformation buffer I (TfB I) and left on ice for 15 minutes. After repelleting, the cells were resuspended in 4ml of transformation buffer II (TfB II) and left on ice

for 20 minutes. Aliquots (200µl) were snap frozen on dry ice/ethanol and stored until required at -70°C.

psi broth: 2% w/v tryptone, 0.5% w/v yeast extract, 20mM Mg_2SO_4, 10mM NaCl, 5mM KCl

TfB I: 15% v/v glycerol, 200mM $MnCl_2$, 100mM RbCl, 10mM $CaCl_2$, 35mM NaOAc, pH 5.9

TfB II: 15% v/v glycerol, 10mM RbCl, 80mM $CaCl_2$, 10mM MOPS, pH 6.8 with KOH

2.1.3.2 Transformation

An aliquot (200µl) of competent bacteria was thawed on ice and incubated for 30 minutes with 50-200ng DNA. The cells were then heat shocked at 42°C for 60-90 seconds, followed by brief cooling on ice. LB (800µl) was added, and the cells incubated at 37°C for 60 minutes. During this period the selective marker, the ampicillin resistance gene, is expressed. The cells were then pelleted by centrifugation at 12000g for 30 seconds, resuspended in 100µl LB, and spread onto LB/amp plates. After overnight incubation, single colonies were picked for subsequent analysis.

Transformation of the commercially prepared DH5α cells was carried out as above, but with slight modifications. The amount of DNA added to the cells was less (1-20ng), reflecting the greater efficiency for transformation of this strain. Heat shock was performed at 37°C for 20 seconds.

2.1.4 Bacterial Stocks

A single colony of transformed bacteria was inoculated into 5ml LB/amp, and incubated for 16 hours. Aliquots of the saturated culture were made up to 20% v/v sterile glycerol, and frozen in dry ice/ethanol for 10 minutes. These were transferred to -70°C for long term storage.

2.2 Tissue Culture

2.2.1.1 Cell Lines

Ovine fibroblasts used as the host cells for viral infection were obtained from MVV negative sheep, by the biopsy method of Rheinwald and Green (1977) and were a kind gift of Dr. B.A. Blacklaws, Department of Veterinary Pathology, University of Edinburgh. Simian COS-1 cells, used for Rev functional assays, were a kind gift of Dr. G. Entrican, Moredun Research

Institute, Edinburgh.

2.2.1.2 Media and Culture Conditions

Cells were maintained as adherent monolayers in plastic flasks (Nunclon, Denmark) and incubated at 37°C in an atmosphere of 5% CO_2, unless otherwise stated. The growth medium used was DMEM, supplemented with 2mM L-glutamine, 100μg/ml streptomycin, 100U/ml benzylpenicillin, 2.5μg/ml fungizone. Foetal calf serum (FCS) was added as required, typically to 10% v/v. As used here, 10%FCS/DMEM refers to fully supplemented DMEM containing 10% FCS.

2.2.1.3 Cell Passage

Cells were grown to near confluence and washed twice with versene (0.5mM EDTA, 0.0013% w/v phenol red). Incubation for 2 minutes in trypsin-versene (0.05% w/v trypsin in versene) led to removal of the cells from the flask surface. The cell suspension was diluted with 1/10 volume FCS to inhibit further trypsin digestion, and centrifuged at 1500g for 2 minutes. The pelleted cells were washed in 10%FCS/DMEM, and then seeded into fresh flasks at a dilution factor of 1:4 (fibroblasts) or 1:20 (COS-1).

2.2.1.4 Estimation of Cell Concentration

A small volume (10μl) of cell suspension was diluted 1:1 in Trypan Blue stain. This was applied to a haemocytometer chamber. Viable cells (unstained) were counted in each of four divisions (total = **n**). The cell concentration was $2\mathbf{n}/4 \times 10^4$/ml.

2.2.2 Virus Infection

Ovine fibroblasts were grown to near confluence, and the media removed. Virus was diluted in 20ml of DMEM, and added to the flasks at an approximate multiplicity of infection of one. The flasks were incubated at 4°C for 60 minutes to allow adsorption of virus to cellular receptors. Transfer to 37°C resulted in synchronous infection of the fibroblasts. The medium was removed after 1 hour, replaced with 2%FCS/DMEM and incubation continued. Cells were harvested when displaying significant syncytia formation (viral cytopathic effect).

2.3 Common Reagents in Molecular Biology

(1) Sterile Phosphate Buffered Saline (SPBS): 137mM NaCl, 26.8mM KCl, 14.7mM KH_2PO_4, 81mM Na_2HPO_4.

(2) TAE: 40mM Trizma base, 0.114% w/v glacial acetic acid, 1mM EDTA

(3) TBE: 90mM Trizma base, 90mM boric acid, 1mM EDTA

(4) TE: 10mM Tris.HCl pH 8, 1mM EDTA

(5) TEN: 10mM Tris.HCl pH 7.4, 2mM EDTA, 140mM NaCl

2.4 Polymerase Chain Reaction

2.4.1 Preparation of template DNA

DNA from virally infected cells was used as a template for PCR. Infected cells were removed from flasks by agitation with sterile glass beads (4.5-5.5mm, BDH) and washed twice in SPBS. Cells were pelleted by centrifugation at 1500g for 5 minutes, and resuspended in 500µl lysis buffer (0.5% w/v SDS, 1M NaCl, 1mM EDTA, 50mM Tris.HCl pH 8). Proteinase K was added to a final concentration of 100µg/ml, and the reaction incubated at 68°C for 1 hour. DNA was purified by hot phenol extraction (2.5.2), precipitated in ethanol, washed in 70% ethanol, and resuspended in 500µl TE. RNA contaminants were removed by incubation with RNase A at 50µg/ml for 30 minutes at 37°C. A further round of phenol extraction and ethanol precipitation yielded the purified DNA, which was resuspended in 200µl dH_2O. A single T175 flask (~10^7 fibroblasts) yielded ~100µg DNA.

2.4.2 Primers

Primers were synthesised by Oswel DNA Service, Department of Chemistry, University of Edinburgh, or by the oligomer synthesis unit, Howard Hughes Medical Center, Duke University, North Carolina. Primers were designed according to certain criteria. Typically, primers were 18-30 nucleotides in length. Complementary sequences within a primer were avoided, so as to reduce the possibility of primer-dimer formation. A 3' terminal G or C residue was included. To harmonise annealing temperatures, a 50% GC content was maintained.

2.4.3 Reaction parameters

PCR reactions were carried out in 100µl volumes using sterile 0.5ml eppendorf

tubes. Reactions were performed in Ohara buffer (3mM DTT, 50mM KCl, 1.5mM $MgCl_2$, 10mM Tris.HCl pH 8.8; Ohara, 1989) with 10μg BSA, 200μM of each dNTP, DNA template (1μl Cell DNA, ~500ng), primers (10-50 pmoles) and 2 units *Thermus aquaticus* (Taq) DNA polymerase enzyme. Mineral oil (100μl) was added as a covering layer to prevent evaporation. Thermal cycling was performed over 35 cycles using a Techne Programmable Driblock PHC-1 machine. Each cycle had the following parameters: denaturation at 95°C/30 seconds, annealing at 55°C/30 seconds, extension at 72°C/60 seconds. A final extension step at 72°C for 5 minutes was included to allow completion of partially extended strands. The reaction outcome was investigated by agarose gel electrophoresis of 10μl of reaction mix. When necessary, completed reactions were stored at -20°C before analysis.

2.5 Purification of Nucleic Acids

2.5.1 Phenol/Chloroform Extraction

DNA was routinely purified by phenol/chloroform extraction, followed by ethanol precipitation. Phenol was prepared by equilibration of an aliquot of redistilled phenol with 0.1M Tris.HCl pH 8.0, and the addition of 0.05% w/v 8-Hydroxyquinoline. Equilibrated phenol was stored in the dark at 4°C for periods of up to 4 weeks. To extract DNA, an equal volume of phenol was added to the sample and mixed by vortexing. The solution was centrifuged at 12000g for 1 minute to separate the aqueous and non-aqueous components. The upper, aqueous layer was removed to a fresh tube. This procedure was repeated with phenol/chloroform (1/2 volume of each), and with chloroform. RNA was extracted similarly, using phenol equilibrated to pH 5 with NaOAc. The term 'phenol extracted' will refer to this entire procedure.

2.5.2 Hot Phenol Extraction

DNA isolated by electrophoresis in low gelling temperature agarose (lgt), was purified by hot phenol extraction. Lgt containing the isolated DNA was melted at 65°C for 10 minutes. An equal volume of dH_2O was added to disrupt resetting of the gel. The sample was then extracted with 1ml phenol, preheated to 65°C. Subsequent extraction was carried out as above (2.5.1).

2.5.3 Purification with silica

DNA was purified by absorption to a modified silica matrix using the Geneclean II™ kit (Stratech). The manufacturer's instructions were followed throughout. Briefly, the protocol involved the removal of contaminants by the selective binding of DNA to the silica matrix in chaotropic conditions (4M NaI). The bound DNA is washed with NaCl/ethanol, and eluted in dH_2O or a low salt solution. DNA molecules shorter than ~500bp may possess sufficiently high avidity to the silica to result in a low yield on elution. Thus, this method was only used for molecules >500bp in size.

2.5.4 Ethanol Precipitation

Nucleic acids purified by the above methods were precipitated by addition of 3M NaOAc pH 5.2, to 0.25M, and 2.5 volumes of nucleic acid grade ethanol, with incubation at -20°C for 30-90 minutes. The precipitated material was collected by centrifugation at 12000g for 10 minutes, washed with 70% v/v ethanol, and air dried. The pellet was resuspended in an appropriate volume of dH_2O or TE (for RNA, RNase free dH_2O), and stored at -20°C.

2.6 Restriction Digests

DNA samples for analysis or enzymatic manipulation were digested using the appropriate restriction endonuclease. Typically, 1-5µg of DNA was digested with 10U of enzyme in manufacturer's buffer for 1 hour at 37°C. A second aliquot of enzyme was then added, and incubation continued for 2-3 hours. Where double digests were required, the buffer used was chosen to give greatest activity for both enzymes, using published activity tables (Promega).

2.7 DNA Cloning

2.7.1 Preparation of DNA for Cloning

Vector and insert were digested with the appropriate restriction endonuclease(s). The vector was dephosphorylated, to reduce the incidence of self ligation, by the addition of 1U of calf intestinal phosphatase (cip) and incubation at 37°C. After 30 minutes a second aliquot of cip was added, and incubation continued for 30 minutes. The vector and insert were purified

using a technique appropriate to the size of the DNA (2.5) and finally resuspended in dH_2O. PCR products were initially cloned into the pCR™II vector, a component of the TA Cloning Kit (InVitrogen, USA), by following the manufacturer's instructions.

2.7.2 Ligation

Ligation reactions were typically carried out using 100ng of vector DNA, and an approximate three fold molar excess of insert DNA. Where this was unsuccessful, a range of insert to vector ratios from 1:3 to 3:1 was used. Ligations were performed in small reaction volumes (10-20µl), using 5U T4 DNA ligase and the manufacturer's buffer. Reactions were incubated at 12°C for 16 hours. A vector only control was included to allow determination of cip efficiency.

2.8 Agarose Gel Electrophoresis

DNA samples were analysed on 1-1.5% w/v agarose/TAE gels containing 0.6µg/ml ethidium bromide. Prior to loading, samples were mixed with 1/6 volume 6x DNA loading buffer (14% w/v Ficoll, 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol). All gels were run in 1xTAE buffer, at 80-100V. An UV transilluminator was used to visualise DNA bands. Preparative agarose gels were run as above, with several modifications. DNA samples were run in 1.5% low gelling temperature agarose/TAE gels. Electrophoresis was carried out at 50-60V at 4°C to prevent melting of the agarose. Each sample was run on a separate gel, to avoid potential cross-contamination. The DNA to be purified was located by UV transillumination and excised in a minimum of agarose, using a clean scalpel blade.

2.8.1 DNA Markers for Agarose Gel Electrophoresis

HindIII and EcoRI digested λ DNA (NBL), with fragments of sizes: 21226, 5146, 4973, 4268, 3530, 2027, 1904, 1709, 1375, 947, 831, 564 and 125 base pairs.

2.9 Small Scale Purification of Plasmid DNA

A single colony of transformed bacteria was inoculated into 5ml LB/amp, and incubated for 16 hours. Plasmid DNA was purified by a modification of the alkaline lysis technique of Birnboim and Doly (1979). The saturated culture was centrifuged at 12000g for

30 seconds, and the pelleted bacterial cells resuspended in 1 volume (typically 200µl) of ice cold lysis buffer (50mM glucose, 10mM EDTA, 25mM Tris.HCl pH8.0). After incubating at room temperature for 5 minutes, 2 volumes (400µl) of freshly prepared alkaline SDS (0.2M NaOH, 1% w/v SDS) were added with gentle mixing by inversion. Addition of the alkaline SDS solution leads to cell lysis and denaturation of the nucleic acids and proteins. Following incubation on ice for 5 minutes, 1.5 volumes (300µl) of potassium acetate pH4.8 was added, and incubation on ice continued for a further 10 minutes. The neutralisation of the solution brought about by the addition of the potassium acetate leads to selective renaturation of the covalently closed, circular plasmid DNA: the chromosomal DNA, protein and high molecular weight RNA remains denatured and is precipitated as a complex by the high salt concentration. Centrifugation for 10 minutes at 12000g leads to pelleting of the precipitated material. The supernatant was decanted to a fresh eppendorf tube. Plasmid DNA was extracted with phenol and precipitated in ethanol. After pelleting, washing in 70% ethanol, and resuspension in dH_2O, the remaining RNA was removed by the addition of DNase free RNase A to a final concentration of 20µg/ml, with incubation at 37°C for 60 minutes. After a second round of phenol extraction and ethanol precipitation, the purified plasmid DNA was finally resuspended in a small volume of dH_2O. Typically, 2ml of bacterial culture yielded 3-5µg of plasmid DNA.

2.10 Large Scale Purification of Plasmid DNA

A single colony of transformed cells was inoculated into 10ml LB/amp and incubated for 16 hours. The preculture was then diluted 1:100 into 500ml LB/amp, and regrown to saturation (12-16 hours). Chloramphenicol amplification was used for certain plasmids which gave a poor yield with this growth regime. The preculture was diluted 1:250 into 25ml LB/amp and incubated until late log phase (OD_{600} ~ 0.6) was reached. This culture was then diluted 1:20 into 500ml LB/amp and incubated for 2.5 hours, at which point the OD_{600} had reached 0.4. Chloramphenicol was added to 170µg/ml (2.5ml of a 34mg/ml stock solution in ethanol) and incubation continued for a further 12-16 hours. Cells were pelleted by centrifugation in a bench top centrifuge at 3000g for 10 minutes.

Plasmid DNA was purified using the Qiagen Plasmid Kit (Hybaid Ltd, London) following the manufacturer's instructions. In outline, a supernatant containing plasmid DNA is generated by an alkaline lysis method which is essentially a scaled up version of that detailed

above (2.9). An ion exchange resin binds specifically to plasmid DNA, allowing contaminants to be removed. The isolated DNA is eluted in high salt conditions. The DH5α strain of *E. coli* was used as the host strain of choice for plasmids purified by this procedure, as suggested by the manufacturer.

2.11 DNA Sequencing

The di-deoxy chain termination method (Sanger *et al*, 1977) was used as the basis for DNA sequencing.

2.11.1 Sequencing Reactions

Double stranded sequencing was performed using an alkaline denaturation protocol and the Sequenase Version 2.0 kit (USB Corporation). High template purity is required for double stranded sequencing, thus template plasmid was prepared by Geneclean treatment of plasmid minipreparations, or by Qiagen column chromatography. Plasmid DNA (2-5μg in 20μl dH_2O) was denatured by addition of 2μl denaturation buffer (2M NaOH, 2mM EDTA) and incubated at 25°C for 5 minutes. The denatured DNA was neutralised with 6.5ml 3M NaOAc, and precipitated at -20°C by the addition of 7μl dH_2O and 75μl ethanol. Pelleted DNA was washed with 70% ethanol and resuspended in 7μl dH_2O, 2μl 5X sequenase buffer and 1μl primer. An approximate 1:1 template:primer stoichiometry was used. To anneal primer and template, the solution was incubated at 65°C for 2 minutes and either snapped cooled on ice, or cooled to 35°C over a period of 25-30 minutes. Sequencing reactions were then performed with the Sequenase kit using the manufacturer's instructions. Briefly, 1μl 0.1M DTT, 2μl 1:5 diluted labelling mix, and 0.5μl $\alpha^{35}S$ dATP (specific activity 400Ci/mmol) were added to the reactions along with 2μl 1:8 diluted Sequenase Version 2.0 (a modified T7 DNA polymerase) and incubated at 25°C for 5 minutes. To terminate the reactions, 3.5μl of each mix was added to each of four tubes containing 2.5μl termination mix (a,c,g,t) and incubated for a further 10 minutes at 37°C. Finally 4μl of stop solution was added to each tube. Reactions were resolved immediately, or stored at -20°C for short periods.

2.11.2 Sequencing gels

Sequencing reactions were resolved by denaturing polyacrylamide gel

electrophoresis. Gel mix consisted of 6% w/v acrylamide (29:1 acrylamide:bisacrylamide), 8M urea, 0.5X TBE. The Sequi-Gen apparatus (Biorad) was used. The sequencing plates were sealed at the base with 50ml gel mix, 150µl TEMED, 210µl 25% APS. For the main gel, gel mix (150ml) was filtered at 45µm and degassed under vacuum; 150µl 25% APS and 150µl TEMED added and the solution poured. Gels were pre-run in 0.5X TBE until the gel temperature reached 50-55°C. The reactions were heated to 75°C for 3-5 minutes and 2µl loaded onto the gel. Gels were run at 2500-3000 volts for 2-6 hours. The gels were then laid onto Whatman 3MM paper and dried at 80°C for 2.5 hours under vacuum on a Bio-Rad Model 583 gel drier. Autoradiography was carried out by exposure to Kodak X-OMAT R film for 1-8 days.

2.12 Production of Recombinant Proteins in Yeast

2.12.1 Yeast Growth Media and Conditions

(1) YEPD: 2% w/v peptone, 2% w/v glucose, 1% w/v yeast extract

(2) Synthetic complete-glucose medium (Sc-glc): 0.67% w/v yeast nitrogen base without amino acids, 1% w/v glucose

(3) Sc-glc/gal: As (2) above, but with 0.3% w/v glucose, 1% w/v galactose

(4) Amino acids: leucine (leu), tryptophan (trp) and uracil (ura) added to media as required at 20mg/ml

(5) Agar plates: Medium supplemented with 2% w/v nutrient agar

(6) TEN buffer with protease inhibitors (TEN/pi): 1µg/ml each antipain, chymostatin, leupeptin, pepstatin A (stock solutions in dimethyl sulphoxide, DMSO) and aprotinin (in dH_2O) and 0.5mM PMSF (in ethanol) in TEN buffer

(7) Acid washed glass beads: Beads (40 mesh, BDH) washed once in concentrated sulphuric acid, rinsed 10 times in H_2O and 10 times in dH_2O, air dried and baked at 150°C for 2 hours

(8) Dialysis tubing: prepared by boiling for 3x3 minutes (1x in 1mM EDTA, 2x in dH_2O)

Incubation of yeast cultures was performed at 30°C with vigorous shaking on a flat bed rotator (New Brunswick Scientific) at ~260rpm. Culture densities were measured by spectroscopy at OD_{600}. For strain BJ2168, an OD_{600} of 1 is equivalent to ~$3x10^7$ cells/ml.

2.12.2 Yeast strains

The protease deficient strain BJ2168 (a, leu2, trp1, ura3, prb1, pep4, pcr1, gal2) was used as the host cell for expression of native and recombinant Ty-vlps. A second strain, the diploid MC5 (prb1, prc1, pep4, trp1, ura3, leu2) was used in an attempt to circumvent problems with recombinant protein toxicity.

2.12.3 Transformation of Yeast

Yeast cells were transformed using the lithium acetate based protocol developed by Ito *et al.*(1983).

2.12.3.1 Generation of Competent Cells

Untransformed yeast of the appropriate strain were inoculated into 100ml YEPD/leu and grown to an OD_{600} of ~0.7. Cells were harvested by centrifugation at 3500g for 5 minutes and washed once with 10ml yeast transformation buffer (YTB, 0.1M LiOAc, 1xTE, pH 7.5). After repelleting, the cells were incubated for 30 minutes at 30°C in 20ml YTB. The cells were pelleted once again, and finally resuspended in 1ml YTB. Competent cells were divided into 100µl aliquots and used immediately, or adjusted to 20% v/v glycerol and stored at -70°C.

2.12.3.2 Transformation

DNA (2-5µg each plasmid, 20µg boiled salmon sperm DNA as carrier) was added to an aliquot of competent yeast cells. After incubation at 30°C for 30 minutes, 0.5ml YTB supplemented with 40% w/v polyethylene glycol (PEG_{4000}),was added and incubation continued for a further 60 minutes. The cells were heat shocked at 42°C for 5 minutes, before plating out onto Sc-glc/agar plates containing selective nutrients. The plates were incubated until colonies became visible (4-14 days).

2.12.3.3 Storage of Yeast Transformants

To generate stocks of transformants, a single colony was inoculated into 50ml of Sc-glc with appropriate amino acids, and incubated until a cell density of 2-4x107 had been reached. Glycerol was added to a final concentration of 20% (v/v) and 1ml aliquots frozen at -20°C for two hours before transferring to -70°C for long term storage (9-12 months).

2.12.4 Analysis of transformants

2.12.4.1 Constitutive Expression

Sc-glc/trp/ura (50ml) was inoculated with a single colony of yeast transformed with a pMA5620 derived plasmid (T.A), and the culture incubated until an OD_{600} of 0.8-1 was reached (2-3 days). The cells were pelleted by centrifugation at 3500g for 5 minutes and resuspended in 1ml TEN buffer. Breakage of the cells was achieved by 3x1 minute periods of vortexing with 1g acid washed glass beads. Cell lysates were collected and analysed by SDS-PAGE and Western blotting.

2.12.4.2 Inducible Expression

Yeast transformed with a pOGS40 derived plasmid were grown to an $OD_{600}$of 0.8-1 in Sc-glc/trp. After pelleting as above, the cells were resuspended in 50ml Sc-glc/gal/trp, and incubation continued for a further 24 hours. Subsequent steps were carried out as above.

2.12.5 Large scale production

2.12.5.1 Constitutive Production of Native p1 Ty-vlps

Sc-glc/trp/ura (200ml) was inoculated with a single stock of T.A cells (transformed with pMA5620). The culture was incubated to an OD_{600} of 1-1.2. 50ml of this culture was diluted into each of 4x1L Sc-glc/trp/ura in 2L conical flasks, and incubation continued for 24 hours. The cells were harvested by centrifugation in a J2-21 rotor (Beckman) at 5000g for 5 minutes at 4°C. Pellets were washed twice with dH_2O and once with TEN, and then were processed immediately or stored at -20°C.

2.12.5.2 Inducible Production of Recombinant p1:Rev Ty-vlps

2.12.5.2.1 Original Protocol

A preculture of TRev.U cells was generated, as above, in Sc-glc/trp. The preculture (50ml) was inoculated into 1L of Sc-glc/trp in 2L conical flasks, and grown to an OD_{600} of ~1.5. This culture was then split into 4x1L Sc-glc/gal/trp, and incubation continued for 24 hours. Cells were harvested as above.

2.12.5.2.2 Modified Protocol

A modified protocol was established in an attempt to reduce the effect of toxicity of expression of p1:Rev. Four yeast stocks were used to inoculate 100ml of Sc-glc/trp. After

growth to OD_{600} of ~1, the preculture (50ml) was inoculated into each of 2x400ml Sc-glc/trp. This culture was grown to an OD_{600} of ~1, and split into 8x500ml Sc-glc/trp +/- 0.2% yeast extract. Cultures were incubated for 24 hours, and then expression was induced by pelleting of cells by centrifugation at 5000g for 5 minutes, and resuspension in an equal volume of Sc-glc/gal/trp. Induction was carried out for 7 hours, and cells harvested as above.

2.12.6 Preparation of Ty-vlps

Cell pellets were removed to 4x30ml Corex tubes, and resuspended in 4ml TEN/pi buffer. Acid washed glass beads (5ml) were added, and the cells broken by vortexing for 10x 30 second periods, with one minute intervals of cooling on ice. The cell suspensions were centrifuged at 2000g for 5 minutes, and the supernatants collected. Pellets were resuspended in TEN/pi and the above procedure repeated until greater than 80% cell breakage had been achieved (by microscopic examination). Supernatants were pooled and centrifuged at 13000g for 20 minutes at 4°C, to remove cellular debris. Ty-vlps were concentrated by pelleting onto a sucrose cushion. The supernatant was layered onto 2ml 60% w/v sucrose/TEN in 30ml Corex tubes and centrifuged at 100,000g for 1 hour at 4°C. The cushion and interface layer containing the Ty-vlps, were collected and the sucrose removed by overnight dialysis against fresh TEN buffer, or by sephadex chromatography (2.12.8).

2.12.7 Purification of Ty-vlps

2.12.7.1 Purification by Density Gradient Centrifugation

A 15-45% linear sucrose gradient was formed by sequential layering of 8ml volumes of 45%, 35%, 25%, and 15% w/v sucrose in TEN buffer, in a 40ml centrifuge tube, with overnight equilibration. A 2ml 60% sucrose cushion was applied immediately before use. Ty-vlp containing material (4ml) was added to the top of the gradient, and the tube centrifuged at 100,000g for 3 hours at 4°C. The gradient was harvested as 2ml fractions, and the fractions were analysed for Ty-vlp content by SDS-PAGE and Western blotting. A second round of centrifugation was performed where necessary.

2.12.7.2 Purification by Affinity Gel Chromatography

2.12.7.2.1 Preparation of Affinity Gel Column

Required volume (2ml) of Affigel-10 (Biorad) was removed from an isopropanol suspension and washed twice in ice-cold water. Protein coupling was performed by incubating the purified γ-globulin fraction of rabbit anti-p1 antiserum (αp1; 2.23.2) with affigel-10 with gentle agitation at 4°C overnight. Free antibody was removed by decanting the supernatant after centrifugation of the affigel-10 for 5 minutes at 1500g. Unoccupied protein binding sites in the affigel-10/αp1 were blocked by incubation with 0.2ml 1M ethanolamine (pH 8)/ 1ml phosphate buffer/0.05% sodium azide for one hour at 25°C. The affigel-10 suspension was washed three times with PBS/0.05% sodium azide by centrifugation, resuspension and mixing. Washed affigel-10/αp1 was loaded into a 10x1cm Econo-chromatography column (Biorad), and stored until use in PBS/0.05% sodium azide.

2.12.7.2.2 Purification

Immediately prior to adsorption of antigen, the affigel-10/αp1 column was washed with 15mM triethanolamine/0.5% sodium deoxycholate (TEA/DOC; pH 8) for 30 minutes, followed by TEA/DOC/0.5M NaCl pH 11.3 for a maximum of 30 minutes. The column was then washed once in PBS/0.05% sodium azide and once in TNT (20mM Tris, 140mM NaCl, 0.5% Triton X-100, pH 8). The p1:Rev gradient fractions to be purified were adsorped onto the column over 24 hours by recirculation using chromatography tubing. The column was washed once with TNT and once with TEA/DOC, before elution of specifically bound material in TEA/DOC/NaCl pH 11.3. The eluate was collected as 1ml fractions, with each fraction neutralised by the addition of 0.3ml 0.5M TEA pH7.9. The fractions were analysed for p1:Rev content by SDS-PAGE and western blotting.

2.12.8 Removal of Sucrose by Sephadex Chromatography

Sephadex G-25 (Sigma) was swollen in TEN/pi and ~5ml applied to a chromatography column. After washing with 2x20ml TEN/pi, sucrose-containing material was added. After the bed volume of liquid had run through the column, samples were collected as 2ml aliquots. These were analysed for protein content by SDS-PAGE and protein concentration assay, and for sucrose content by use of a refractometer (Mackay and Lyn, Edinburgh), using 5% sucrose as standard.

2.13 Production of Recombinant Proteins in Bacteria

2.13.1 Small Scale Production and Purification of GST Fusion Proteins

Small scale purification of GST fusion protein for analysis was achieved by following the protocol of Smith and Johnson (1988). A single colony of bacteria transformed with the recombinant plasmid was inoculated into 2ml LB/amp and incubated until visibly turbid (3-5 hours). Fusion protein expression was induced by the addition of IPTG to 0.1mM, with further incubation for 2-3 hours. The cells were pelleted by centrifugation at 12000g for 30 seconds, and the pellet resuspended in 500µl ice cold SPBS. The cells were lysed by sonication using a Dawe 2mm probe sonicator in a single 10-15 second burst of sonication with immersion of the probe tip well within the solution to prevent frothing. Centrifugation at 12000g for 5 minutes at 4°C removed the cellular debris and intact cells. The supernatant was removed to a fresh eppendorf tube. The fusion protein was purified by addition of 50µl of a pre-swollen 50% slurry of glutathione-agarose beads (Sigma) which selectively bind to the GST moiety. The tubes were mixed on an orbital shaker for 5 minutes at room temperature. The beads were collected by centrifugation at 1500g for 1 minute, and washed three times with SPBS. Finally, the beads were resuspended in an equal volume (50µl) of SDS-PAGE sample buffer, and analysed on a 10% SDS polyacrylamide gel.

2.13.2 Large Scale Production and Purification of GST Fusion Protein

Large scale purification of fusion protein for use in functional assays was performed in an essentially similar manner to the protocol outlined above, but by using scaled up volumes. A single colony of pGEX transformant was inoculated into 100ml LB/amp and incubated for 12-16 hours. This culture (50ml) was then diluted 1:10 into 500ml LB/amp and incubation continued for 1 hour. IPTG was added to 0.1mM and incubation continued for 3-4 hours. After induction, the culture was centrifuged at 5000g for 10 minutes in a Beckman JA-10 rotor at 4°C. Cells were resuspended in 10ml ice cold SPBS. 2 x30 second bursts of sonication were separated by a 1 minute interval of cooling on ice. Triton X-100 was added to 1% v/v in order to discourage interactions between the fusion protein and contaminating bacterial proteins. The samples were centrifuged at 10000g for 5 minutes in a Beckman JA-20 rotor at 4°C. The supernatant was removed to a 15ml plastic tube, and 500µl of a 50% slurry of glutathione

agarose beads added. Following gentle mixing at room temperature for 5-10 minutes, the beads were collected by centrifugation at 1500g in a bench top centrifuge and washed three times with 15ml SPBS. The beads were finally resuspended in 1.5ml of SPBS and transferred to an eppendorf tube.

2.13.3 Elution of Fusion Protein from Agarose Beads

The beads were resuspended in 500µl elution buffer (10mM reduced glutathione, 50mM Tris.HCl, pH 8.0). Tubes were mixed gently for 5 minutes at room temperature, centrifuged at 1500g for 1 minute and the supernatant collected. Elution was repeated twice. Protein eluates for use in functional assays were made up to 10% v/v glycerol, 50mM NaCl, 10mM KCl, 2mM DTT, 0.25mM PMSF, and stored in 100ml aliquots at -70°C for up to 6 weeks before use.

2.14 Protease Cleavage of Fusion Proteins

Fusion protein samples for cleavage were adjusted to the appropriate conditions for protease activity. Ty-vlps were dialysed into factor Xa buffer (100mM Tris.HCl pH 7.6, 10mM $CaCl_2$) and 0.05% w/v sodium deoxycholate, 0.05% w/v CHAPS (3-(3-cholamidopropyl) dimethylammonio-1-propanesulphonate). GST fusion protein eluates were adjusted to 1mM $CaCl_2$ for factor Xa buffer, 2.5mM for thrombin buffer. Thrombin or activated factor X was added at a range of concentrations (0.1-5% w/w) and the reactions incubated at 25°C for 2 hours. Reaction outcomes were analysed by SDS-PAGE and Western blotting.

2.15 Analysis of Proteins

2.15.1 Gel Electrophoresis

Proteins were analysed by discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions, using a modification of the method of Laemmli (1970). A stock solution of 30% w/v acrylamide:0.8% w/v bisacrylamide was prepared, degassed under vacuum, and stored at 4°C for up to 6 weeks. To prepare separating gels, the stock solution was diluted to the required concentration (typically 10%). The gel mix additionally contained 0.1% SDS, 1.3mM EDTA, 0.26M Tris.HCl pH 8.7. Gels were polymerised with 0.5% w/v APS and 0.05% v/v TEMED, and cast into the gel mould.

Gradient gels were made by the mixing of two different percentage acrylamide solutions using a Watson Marlow peristaltic pump. A volume of water was overlaid in order to ensure formation of a smooth gel interface. When the separating gel had set, a layer of stacking gel was applied. This consisted of 3.48% acrylamide, 0.12% SDS, 0.145M Tris.HCl pH 6.8, 0.05% APS, 0.1% TEMED. Wells for sample loading were made by indentation of the stacking gel with a multitooth comb. Protein samples for SDS-PAGE analysis were prepared by mixing with an equal volume of SDS-PAGE sample buffer (15% v/v glycerol, 0.05% SDS, 0.005% w/v bromophenol blue, 150mM β-mercaptoethanol, 50mM Tris.HCl pH 6.8) and boiling for 3 minutes. The samples were loaded onto the gel using a thin gauge Hamilton pipette, and electrophoresis performed at 200V 25mA for 60-90 minutes in running buffer (0.156M glycine, 0.1% SDS, 25mM Trizma base).

2.15.2 Concentration of Proteins by Acetone Precipitation

Where necessary, protein samples to be analysed by SDS-PAGE were concentrated by acetone precipitation. Protein sample was incubated at -20°C for one hour with five volumes acetone. The precipitate was pelleted by centrifugation at 13000g for one minute, and resuspended in an appropriate volume of SDS-PAGE sample buffer.

2.15.3 Visualisation of Proteins Resolved by SDS PAGE

Once electrophoresed, gels were rinsed with dH_2O and either stained for visualisation, or electroblotted for Western blot analysis. The expected protein concentrations determined which stain was used. Coomassie blue stain has an approximate detection limit of 50ng per protein band, staining with silver nitrate a limit of 5ng. After staining the gels were rinsed, and dried onto 3MM paper under vacuum for 2 hours at 80°C.

2.15.3.1 Staining of Gels with Coomassie Blue

Gels were routinely stained with Coomassie brilliant blue G-250 or Coomassie blue R (0.25% w/v Coomassie blue, 20% v/v methanol, 5% v/v glacial acetic acid) for 30 minutes at room temperature, followed by thorough destaining in 20% methanol, 5% glacial acetic acid, to remove dye not bound to protein.

2.15.3.2 Staining of Gels with Silver Nitrate

Gels were fixed by sequential immersion and gentle agitation in (i) 50% methanol, 10% glacial acetic acid for 15 minutes, (ii) 5% methanol, 7% glacial acetic acid for 20-30 minutes, (iii) 10% v/v gluteraldehyde for 20-30 minutes. The gels were then washed in several changes of dH_2O over 16 hours. A modification of the method of Merril *et al* (1981) was used to stain the gels. Gels were stained with 0.1% w/v silver nitrate for 15 minutes, rinsed briefly in dH_2O and the stain developed by addition of 0.05% v/v formaldehyde in 2.6% w/v sodium carbonate solution. To stop the reaction, 2-3 g of solid citric acid was added, followed by thorough rinsing in dH_2O. The stained gels were fixed in 10% v/v Ilfofix (Ilford Lab.) for 1 minute and rinsed in dH_2O.

Gels which had previously been stained in Coomassie blue could be restained in silver nitrate. In this case, the gel was thoroughly rinsed in dH_2O after destaining, and the above protocol followed, beginning with immersion in 10% gluteraldehyde.

2.15.4 Western Blotting

2.15.4.1 Electrophoretic Transfer

After separation by SDS-PAGE, proteins were transferred onto a nitrocellulose membrane using a modification of the technique of Towbin *et al.* (1979). Transfer was performed by placing the gel and nitrocellulose membrane (Hybond C, 0.45μm, Amersham) between sheets of Whatman 3MM paper, soaking with blotting buffer (25mM Tris, 20% methanol) on a Semi Dry Electroblotter (Ancos, Denmark) and electroblotting for 1 hour at 120mA. After transfer, the portion of the membrane containing the molecular weight markers was cut off, stained in 5% w/v amido black and destained in 50% methanol, 5% glacial acetic acid. To prevent non specific binding of probe to empty binding sites on the filter, the nitrocellulose membranes were blocked with 5% w/v skimmed milk/SPBS for a minimum of 12 hours at 4°C.

2.15.4.2 Immunodetection

Blots were probed with primary rabbit antibody diluted in 1% skimmed milk/SPBS for 1 hour at room temperature, with subsequent washing over a 30 minute period with several changes in 1% milk/SPBS. The blots were then incubated with an anti-rabbit IgG alkaline phosphatase linked second antibody, for a further hour, and again washed in 1% milk/SPBS. After equilibration in 0.1M Tris.HCl pH 9.5, the blots were developed by addition of 10ml

development solution (0.2mg/ml NBT (Nitroblue tetrazolium), 0.1mg/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate, diluted in DMF), 200µM $MgCl_2$, 0.1M Tris.HCl pH 9.5). Development was stopped by washing in dH_2O, before the background staining became too pronounced. The blots were air dried.

2.16 Determination of Protein Concentration

Protein concentrations were determined by use of an assay adapted from the Bradford dye binding procedure (Bradford, 1976), supplied by BioRad Lab.Inc. (Hemel Hempstead, Hertfordshire). This is a colorimetric assay based on binding of protein to Coomassie brilliant blue G-250 dye. The assay was performed according to manufacturer's instructions. BSA was used as a protein standard.

2.17 Electron Microscopy

2.17.1 Negative Staining of VLPs

The structure of Ty-vlps was investigated by transmission electron microscopy. A drop of solution containing Ty-vlps was placed on a plastic coated grid. After 15 seconds the grid was washed 3 times with a single drop of dH_2O and allowed to air dry. The sample was fixed in 2% w/v uranyl nitrate and examined.

2.17.2 Preparation of Yeast Cells

Yeast cells were prepared for visualisation by electron microscopy by an adaptation of the method of Byers and Goetsch (1975). Pelleted cells were resuspended in 5ml cacodylate buffer (3% v/v gluteraldehyde, 0.1M cacodylate, 5mM $CaCl_2$, pH 6.8) and incubated at room temperature for 30 minutes. Cells were pelleted, resuspended in 10ml cacodylate buffer, and incubated for 16 hours at 4°C, with rotation. Cells were washed twice with 10ml pre-treatment buffer (0.1M mercaptoethanol, 20mM EDTA, 0.2M Tris.HCl, pH 8.1) and resuspended in 5ml citrate-phosphate buffer (40mM citric acid, 60mM Na_2HPO_4, pH 5.8) containing 250µl glusulase (DuPont Co., USA) to create spheroplasts. The cells were incubated for one hour at 30°C, followed by two washes in 10ml citrate-phosphate buffer, and resuspended in 5ml of the same buffer. The cells were fixed by incubation at 0°C for one hour in 2% w/v osmium tetroxide, rinsed with dH2O, and incubated for a further hour in 2% (v/v) aqueous uranyl acetate. The cells were then dehydrated and embedded in araldite resin. Blocks were

sectioned using a microtome, and viewed by transmission electron microscopy.

2.18 Synthesis of RNA for Functional Assays

2.18.1 Precautions against RNase Contamination

Where possible, all solutions used for experiments involving RNA were made up in RNase free water. This was obtained by treatment of dH_2O with 0.05% v/v DEPC, with incubation for 1 hour at 65°C and for 16 hours at 37°C. The solution was then autoclaved to remove all traces of DEPC. Plasticware for use with RNA was kept solely for this purpose. Manipulation of RNA was performed, where possible, in a laminar flow hood. Gloves were worn throughout, and changed frequently.

2.18.2 Preparation of Template for Transcription

In order to produce transcripts derived only from the insert sequence, template plasmids were linearised by digestion with a restriction endonuclease with specificity for a site at the 3' end of the insert/vector junction. The products of digestion were resolved on a 1% agarose gel, and digested plasmid purified by hot phenol extraction, precipitated in ethanol, and resuspended in RNase free dH_20 at 200-800ng/µl. It was important to purify digested template away from undigested plasmid as this latter can give rise to very long transcripts which can sequester a large proportion of the labelled nucleotide, thus reducing the efficiency of the transcription reaction.

2.18.3 *In Vitro* Transcription

In vitro transcription was performed using a Maxiscript™ *in vitro* transcription kit (AMS Biotechnology Ltd, Witney, Oxfordshire). The reaction components were assembled in the following order; 1/5x transcription buffer, 10mM DTT, 1µg template DNA, 0.5mM ATP,CTP,GTP, 10 U RNase inhibitor (RNasin) , 50µCi α-^{32}P UTP (400 Ci/mmol) and 10U T7 or SP6 RNA polymerase, made up to 20µl in RNase free dH_20. Incorporation of cold UTP into the reaction is discussed in the main text. The reaction was incubated at 4°C for 30 minutes, at which point a second 10U of polymerase was added, and incubation continued for a further 30 minutes. To stop the reaction, RNase free DNase (2U) was added to remove the template, with incubation at 37°C for 15 minutes. RNase free dH_2O was added to 200µl, and the RNA

purified by extraction with phenol (pH5). After precipitation in ethanol, and washing with 70% ethanol, the RNA was resuspended in 200µl RNase free dH_2O. Aliquots (50µl) were stored at -70°C for up to 2 weeks before use.

2.18.4 Analysis of the Transcription Reaction

2.18.4.1 Reaction Efficiency

The efficiency of the reaction was determined by TCA (trichloroacetate) precipitation. 2µl of the purified RNA was diluted into 198µl TE containing 100mg RNase free salmon sperm DNA. After mixing by vortexing, 5µl of this solution was spotted onto a Whatman GF/C glass fibre filter and air dried. This represents the total radioactivity present. Ice cold 10% w/v TCA (1ml) was added to the remainder of the solution, and the RNA precipitated by incubation on ice for 10 minutes. The salmon sperm DNA acts as a carrier for precipitation, due to the small mass of RNA present. The precipitated RNA was collected by filtration under vacuum through a GF/C filter, with washing in 5ml ethanol, and 5ml 10% TCA. The filters were air dried. This represents the radioactivity incorporated into the RNA. Both filters were immersed in scintillation fluid in a scintillation vial, and the radioactivity counted. The proportion of radioactive label incorporated into RNA could thus be determined.

2.18.4.2 Reaction Products

Products of the transcription reaction were analysed by denaturing polyacrylamide gel electrophoresis. An aliquot (2µl) of the purified products was diluted with 3µl of RNase free dH_2O and 5µl of gel loading buffer (80% w/v formamide, 0.1% w/v xylene cyanol, 0.1% w/v bromophenol blue, 2mM EDTA) added. The tube was heated at 85°C for 3-5 minutes to denature the RNA. The sample was then loaded onto a 6% acrylamide/ 8M urea /TBE gel, and electrophoresis carried out for 45 minutes at 200V. The results were visualised by autoradiography of the dried gel.

2.18.4.3 RNA Markers for PAGE

RNA markers were obtained from Gibco/BRL. Fragment sizes were: 1770, 1520, 1280, 780, 530, 400, 280 and 155 bases.

2.19 *In Vitro* Rev Binding Assays

2.19.1 Binding Conditions

Variable amounts of GSTRev protein were incubated with 20,000 precipitable cpm ^{32}P RNA in a sterile 0.5ml eppendorf tube. The reaction also contained RNA binding buffer (20mM Tris.HCl pH 7.5, 50mM KCl), 1mM DTT, 20µg BSA, 15U RNasin, 2µg yeast tRNA, RNase free dH_2O to a total reaction volume of 20µl. The binding reaction was incubated at 25°C for 10 minutes before the addition of ^{32}P RNA, in order to allow non-specific binding of protein to ytRNA. After addition of ^{32}P RNA, incubation was continued for 15 minutes. Where cold competitor RNA was used, this was added to the reaction before the labelled RNA, and incubated for 10 minutes. The outcome of the binding reaction was determined by two methods. The gel retardation assay involved electrophoretic resolution of complexes formed between protein and RNA. A second method was the filter binding assay, where complexes were trapped by filtration through a nitrocellulose membrane, and quantitated by scintillation counting.

2.19.2 Gel Retardation Assay

Protein-RNA complexes were resolved by electrophoresis through 6% nondenaturing polyacrylamide (crosslinked 29:1 with bisacrylamide) gels, using the Biorad Protean II minigel apparatus. Reactions were diluted with an equal volume of loading buffer (15% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol) and 10µl loaded onto the gel. Gels were run at 100V for 4 hours at 4°C. Gels were dried onto 3MM paper, and the results visualised by autoradiography.

2.19.3 Filter Binding Assay

Filter binding assays were based on the method of Carey *et al*, (1983). Complexes were filtered through a nitrocellulose membrane (Hybond C, 0.45µM, Amersham) using a Biorad Slot Blot apparatus. The membrane was soaked in RNA binding buffer, placed onto 3 sheets of Whatman 3MM filter paper, and the apparatus secured under vacuum. The filter was prewetted with 500µl RNA binding buffer. Slots were filled with 200µl RNA binding buffer, and the rev binding reactions added. After filtration, the slots were washed twice with 500µl RNA binding buffer, and the membrane air dried. The membrane was divided into sections

following the slot outlines, and the bound activity determined by scintillation counting.

2.20 Transfection

Transfection of COS cells for assaying transient expression of introduced genes was performed using two methods. DEAE Dextran mediated transfection was based on the method of McCutchan and Pagano (1968). Transfection by calcium phosphate precipitation was based on the original observation of Graham and Van der Eb (1973).

2.20.1 Preparation of Cells for Transfection

Six well plates (35mm diameter, Nunclon) were pretreated with 0.1% w/v gelatin for 1 hour at room temperature. After washing with 10%FCS/DMEM, each well was seeded with $2x10^5$ COS cells. The cells were incubated for 16 hours in 10%FCS/DMEM, at which point the cell monolayers had reached 60-80% confluence.

2.20.2 Preparation of DNA for Transfection

High plasmid purity is required for efficient transfection. All plasmids were prepared by Qiagen column chromatography (2.10) and resuspended in dH_2O at 0.2 to 1 µg/ml. The purity and concentration of plasmid DNA was determined by spectrophotometry, and checked by agarose gel electrophoresis (2.8).

2.20.3 Transfection with DEAE Dextran

Immediately before transfection, media was aspirated, and the cells washed with 2ml prewarmed SPBS. Transfection solutions (1-2µg total DNA, 100µg DEAE Dextran made up to 200µl with SPBS) were added to each well and distributed evenly by gentle tilting of the plate. The plates were incubated for 30 minutes before addition of 10%FCS/DMEM (2ml) supplemented with 2.5µg/ml fungizone (total 5µg/ml) and 100mM chloroquine to each well, and continued incubation for 2.5 hours. Chloroquine is thought to enhance transfection efficiency by inhibiting microsomal vacuole formation resulting in reduced degradation of incoming DNA (Luthman and Magnusson, 1983). Media was removed by aspiration and the cells shocked by addition of fresh 10%FCS/DMEM supplemented with 10% DMSO for 2.5 minutes at room temperature. DMSO may aid transfection by increasing cell membrane permeability. Following aspiration of the shock solution, the cells were incubated for 48 hours

in 2-3ml 10%FCS/DMEM.

2.20.4 Transfection by Calcium Phosphate Precipitation

Cell media was aspirated and replaced with fresh 10%FCS/DMEM 2-4 hours prior to transfection. Each well was treated with 250µl DNA/calcium phosphate precipitate, composed of 125µl 2x DNA precipitation buffer (280mM NaCl, 10mM KCl, 12mM glucose, 1.5mM Na2HPO4, 50mM HEPES, pH 7.05), 125mM $CaCl_2$, 4-6µg total DNA, diluted in dH_2O. The precipitate was formed by the stepwise addition of the dH_2O, DNA, $CaCl_2$ and buffer to a sterile eppendorf tube with gentle mixing, followed by incubation at room temperature for 20 minutes. The treated cells were incubated for 4 hours. In order to increase transfection efficiency, the cells were then shocked by incubation for 1 minute in 15% v/v glycerol (in 1x precipitation buffer). The cells were washed twice in DMEM and incubated for 48 hours in 10%FCS/DMEM.

2.21 Radioimmunoprecipitation

After 48 hours incubation, media was aspirated and cells washed three times with cysteine/methionine free DMEM (cys/met- DMEM). The cells were starved of cysteine and methionine by incubation in 1ml cys/met- DMEM for one hour. Media was again aspirated, and replaced with 500µl cys/met- DMEM supplemented with 50mCi tran^{35}S label (ICN Flow). After 2 hours incubation, the cells were harvested by gentle agitation in 1ml radioimmunoprecipitation assay buffer (RIPA; 0.1% SDS, 1% Triton X-100, 1% w/v sodium deoxycholate, 0.15M NaCl, 1mM EDTA, 0,25mM PMSF, 10mM Tris.HCl pH 7.4). The suspension was transferred to a 1.5ml eppendorf tube, and cell debris removed by centrifugation at 12000g for 20 minutes at 4°C. The cleared cell lysate was frozen at -70°C at this stage before immunoprecipitation.

The lysate was transferred to a fresh tube, and specific antibody added at the required dilution (typically 1:200) for 16 hours at 4°C. The antibody complexes were precipitated by the addition of 50µl protein A Agarose beads (Sigma). The beads were pelleted by centrifugation, washed three times in RIPA buffer, and an equal volume (50µl) of SDS-PAGE sample buffer added. The agarose/ antibody/ antigen complexes were resolved by electrophoresis through 15% denaturing polyacrylamide gels. The results were visualised

by autoradiography of the dried gels.

2.22 CAT Assay

2.22.1 Harvesting of Transfected Cells

After 48 hours incubation, cells were washed once in SPBS. Glass beads (4.5-5.5mm, BDH) were added to each well, and the cells removed from the plates by agitation in 1ml 250mM Tris.HCl pH 8.0. The suspension was collected in a sterile 1.5ml eppendorf tube, and centrifuged at 12000g for 1 minute. The cell pellet was resuspended in 50µl 250mM Tris.HCl pH8.0. Cells were lysed by three rounds of freeze/thawing (freezing in dry ice/ethanol 15 minutes: thawing in a 37°C water bath 5 minutes). Cellular debris and intact cells were removed by centrifugation at 12000g for 5 minutes, and the supernatant collected for analysis.

2.22.2 Assay for CAT activity

CAT activity in cell lysates was determined by liquid scintillation counting, based on the method of Seed and Sheen (1988). Briefly, a sample of lysate (5µl) was mixed with 5µl n-butyryl CoA (5mg/ml), 2µl ^{14}C-chloramphenicol (0.025mCi/ml) and 118µl 250mM Tris.HCl pH 8.0. After incubating at 37°C for 1 hour, the reaction products were extracted by the addition of 250µl mixed xylenes (Aldrich Chemical Co., Gillingham, Dorset), with vigorous mixing. After centrifugation at 12000g for 3 minutes, the upper organic phase containing the acetylated chlramphenicol was removed to a fresh tube, 250µl dH_2O added, and extraction repeated. Finally, 125µl of the organic phase was mixed with scintillant, and counted in a scintillation counter.

2.22.3 β Galactosidase Assay

The reporter plasmid, pSV-β-Gal (Promega), which expresses the enzyme β-galactosidase, was used as an internal control for transfection and cell harvesting efficiency. Enzyme activity in cell lysates was determined using a colorimetric assay. Cell lysate (10µl) was made up to 50µl with dH_2O, added to an equal volume of 2x assay buffer (120mM Na_2HPO_4, 80mM NaH_2PO_4, 2mM $MgCl_2$, 100mM β-mercaptoethanol, 1.33mg/ml ONPG) and incubated at 37°C for 30-60 minutes. The reaction was terminated by the addition of 150µl 1M $NaCO_3$,

and the OD_{420} determined by spectrophotometry.

2.23 Antibody Generation

2.23.1 Immunisation Protocol

An equal volume (0.5ml) of antigen diluted in SPBS was added to Freund's Complete Antigen (Sigma) and the immunogen emulsified by sonication. Dutch albino rabbits were immunised sub-cutaneously with 0.5ml emulsion. Subsequent immunisations, using Freund's Incomplete Antigen, were performed at 10 and 24 days after priming.

Blood samples were taken immediately prior to initial immunisation and at intervals from 24 days subsequently. Samples were obtained by bleeding from the ear vein, and were allowed to clot by incubation at 4°C for 18 hours. Serum was removed from the clot and stored in 500µl aliquots at -20°C.

2.23.2 Purification of Antiserum by Caprylic Acid Precipitation

Serum (10ml) was diluted in the ratio 1:2 with acetate buffer (0.2M acetate, pH4). Caprylic (octanoic) acid (37.5µl/ml serum) was added dropwise, with vigorous mixing. The mixture was incubated at room temperature for 30 minutes, before centrifugation at 15600g for 30 minutes to precipitate impurities. The supernatant was removed, and dialysed against 0.1M phosphate buffer pH7.2 overnight.

CHAPTER THREE

EXPRESSION OF EV1 REV IN THE YEAST TY-VLP SYSTEM

3.1 Introduction

The recent advances in biotechnology which have allowed the expression of heterologous genes in environments which are easily amenable to manipulation, such as bacterial or yeast cells, have had an immeasurable impact on virological research. Systems for the expression of genes often involve inserting the gene into an expression cassette which contains the *cis*-acting signals, such as those for transcription initiation and termination, required for high level expression in the host cell. In addition, where the expression system involves the formation of fusion proteins, the carrier protein possesses certain characteristics which allow for ease of purification. One such fusion protein expression system is the yeast Ty-VLP system. Developed by British Biotechnology Limited (BBL), Oxford, this system was chosen as the means of expression of MVV EV1 Rev protein. Several reported attributes of this system are of benefit when expressing recombinant protein for functional studies. These include high yield and purity of product, a relatively simple purification system involving non-denaturing conditions, and the option of regulatable expression. The nature of the purification process also allows for versatility with respect to the size of the encoded protein.

The Ty-VLP expression system is based on the Ty1 retrotransposon element of the yeast *Saccharomyces cerevisiae*. The majority of the 30-35 Ty elements distributed at variable chromosomal locations within the yeast haploid genome are 5.9kb in length. Each element consists of an unique region containing two open reading frames, *TYA* and *TYB*, surrounded by long terminal repeats (LTRs or 'δ' sequences) of 335bp. Transcription, initiated from inside the 5' δ sequence, produces a 5.7kb transcript which functions both as the major message, and as an intermediate in transposition (Boeke *et al.*, 1985). Translation of this message gives rise predominantly to the product of *TYA*, a 50kDa phosphoprotein, p1. *TYB* is expressed by a low frequency (~3%) frameshift event, induced by a ribosomal stalling/slippage mechanism at the 3' boundary of *TYA*, which results in translation continuing in the +1 reading frame register (Mellor *et al.*, 1986). The resulting product, p3, is a 190kDa fusion protein containing both p1 and *TYB* encoded residues. The *TYB* specific residues contain reverse transcriptase, protease and integrase activity. These proteins assemble into pre-virus-like particles (VLPs). Particles undergo a process of maturation which involves proteolytic degradation of p1 into four smaller derivatives (p2,4,5,6; Adams *et al.*, 1987b). The mature VLPs, which also contain Ty RNA and a $tRNA_{met}$ primer, are located in the cytoplasm and have an approximate diameter

of 60nm. Transposition is completed when a cDNA copy of the Ty RNA is reverse transcribed, and integrates into the yeast genome, often upstream of tRNA genes.

The Ty-VLP expression system is based on the observation that protein p1 alone can self-assemble to form particles without the presence of p3 and subsequent maturation events mediated by p3 protease activity (Adams *et al.*, 1987a). The expression cassette common to the Ty-VLP vectors contains a modified form of *TYA* (d*TYA*), truncated by the formation of a BamHI linker at codon 381. Hence in frame insertion of a foreign gene at the linker region results in the production of a fusion protein which subsequently assembles into hybrid Ty-VLPs, each containing approximately 300 monomers. Morphologically, the hybrid particles are thought to contain a p1 core with spikes of foreign protein protruding from the surface (Adams *et al.*, 1988).

The expression cassettes are contained within two vector species. Both are derived from bacteria/yeast shuttle vectors, allowing genetic manipulation in bacteria followed by expression in yeast cells. Vector pMA5620 (illustrated by figure 3.1a; Adams *et al.*, 1987a) directs constitutive expression of protein from the yeast phosphoglycerate kinase (PGK) promoter (Dobson *et al.*, 1982). Expression from this strong promoter can generate up to 5% of total cellular protein (Mellor *et al.*, 1985). The expression cassette is followed by termination codons in all three reading frames, and by a transcription terminator. The plasmid also contains origins of replication for both hosts. The yeast origin is based on the 2μ plasmid, maintaining the plasmid at 100-200 copies per cell. Transformed cells are selected in bacteria on the basis of an ampicillin resistance gene and in yeast by the presence of the gene LEU2, which renders strains auxotrophic for leucine independent of this amino acid.

The vector pOGS40 (figure 3.1b; Gilmour *et al.*, 1989) is a derivative of pMA5620, containing a modified PGK promoter (PAL), generated by the replacement of the upstream activating sequence (UAS) of the PGK promoter with that of the GAL 1/10 promoter (Kingsman *et al.*, 1990). The hybrid directs high level transcription only in the presence of galactose. Regulatable transcription allows the cell growth and gene expression phases to be uncoupled. Thus the physiological load on the host cell of high level production of a protein without cellular benefit is lessened. This is of particular importance where expression of the gene exerts a toxic effect on the host cell.

The PAL promoter is regulated by two, host encoded, proteins, the activator pGAL4 and its antagonist pGAL80. In glucose based media, the GAL80 protein binds to the

Figure 3.1: Ty-VLP Expression Vectors and the Helper Plasmid pUG41S

The diagram illustrates the two Ty-VLP expression vectors, pMA5620 and pOGS40
Expression from pMA5620 (A) is driven by the constitutive PGK promoter. The pOGS4
vector (B) is regulatable, due to the presence of the hybrid PGK/GAL promoter, PAL
Expression is induced on addition of galactose.
The helper plasmid pUG41S (C) provides high levels of the regulatory protein pGAL4 to th
PAL promoter in a galactose inducible manner.

pPGK: the phosphoglycerate kinase promoter.

pPAL: the hybrid PGK/GAL promoter.

pGAL 1/10: the promoter of the genes GAL 1 and GAL10. Contains elements to caus
repression in glucose and induction in galactose.

UAS-PGK: the upstream activating sequence of the PGK promoter.

UAS-GAL: the upstream activating sequence of the GAL 1/10 promoter.

TYA(d): truncated form of the gene TyA.

PGKt: translational termination codons in three reading frames from the PGK gene.

BamHI: cloning site for insertion of heterologous gene.

GAL4: gene encoding the galactose positive regulatory factor pGAL4

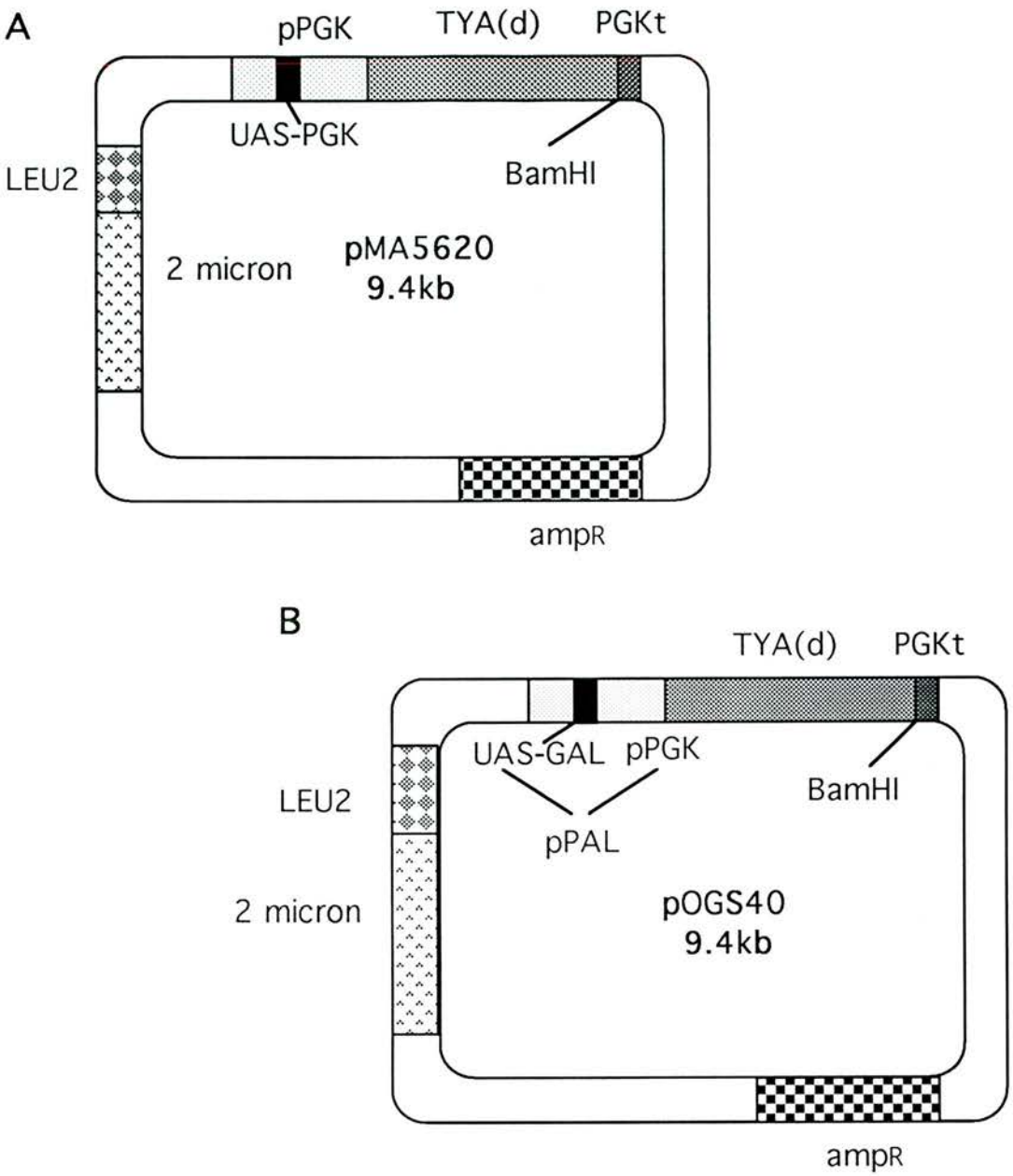
2 micron: replication module for yeast, maintaining plasmid at 100-200 copies per ce

ARS1/CEN4: autonomous replicating sequence for yeast.

amp^R: ampicillin resistance gene.

LEU2: gene for leucine biosynthesis.

URA3: gene for uracil biosynthesis.


A
pPGK
TYA(d)
PGKt
UAS-PGK
BamHI
LEU2
2 micron
pMA5620
9.4kb
ampR
B
TYA(d)
PGKt
UAS-GAL
pPGK
BamHI
pPAL
LEU2
2 micron
pOGS40
9.4kb
ampR
C
pGAL1/10
GAL 4
pUG 41S
~10.8 kb
URA 3
ARS 1/CEN 4

activation domain of pGAL4, preventing its interaction with the UAS-GAL within the PAL promoter (reviewed in Schultz *et al.*, 1987). Only in the absence of glucose and the presence of galactose is the inhibitory effect reversed. Binding of pGAL4 to the UAS-GAL in the context of the GAL 1/10 promoter leads to an approximate 1000-fold increase in expression (St John and Davis, 1979). Thus, the inducibility of this promoter is critically dependent on the relative concentrations of pGAL4 and pGAL80. As the level of pGAL4 within cells may be too low to maximally induce transcription (Johnston and Hopper, 1982), this protein can be supplied by means of a helper plasmid, pUG41S (figure 3.1c). This low copy number plasmid expresses GAL4 under the control of the GAL1/10 promoter, which is itself galactose inducible. Co-transformation of cells with pOGS40 and pUG41S therefore should give rise to greater expression than with pOGS40 alone (Schultz *et al.*, 1987). Cells transformed with pUG41S are selected on the basis of uracil independent growth.

The *S. cerevisiae* strain, BJ2168, has a phenotype with several characteristics required for Ty-VLP expression. It is partially protease deficient. This should reduce possible host-mediated degradation of the expressed protein. It is auxotrophic for three amino acids, leucine, tryptophan and uracil, which is the basis for selection of cells transformed with the Ty-VLP vectors. It should be noted that BJ2168 cells contain a mutation within the GAL2 galactose permease gene, which results in the inability to uptake galactose by an inducible mechanism. However, galactose can enter the cell via a less-efficient constitutive pathway, thus ensuring the inducibility of plasmid pOGS40.

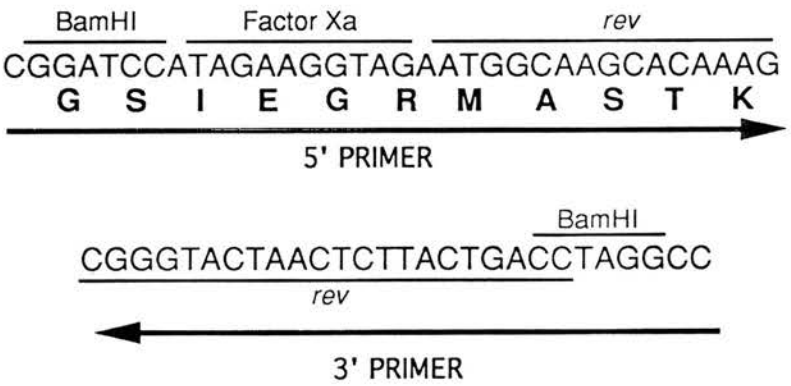
Purification of fusion proteins is described briefly. Cells which have expressed hybrid Ty-VLPs are harvested and lysates prepared. Purification of the Ty-VLPs is based on centrifugation of crude extracts through sucrose density gradients (Burns *et al.*, 1991). The particulate nature of the Ty-VLPs results in relatively tight banding within the gradient. Within a range of approximately 3-40kDa, fusion of a foreign protein to p1 does not significantly alter the sedimentation properties of the Ty-VLPs (Burns *et al.*, 1992). This fact adds to the versatility of the system. Two rounds of sedimentation may be required for enhanced purity. If the foreign protein is required in its native state, it can be removed from the VLPs by the incorporation of a specific protease cleavage site at the boundary between (d) *TYA* and the inserted gene (Braddock *et al.*, 1989).

Proteins which have been successfully expressed in the yeast Ty-VLP system include HIV-1 Tat (Braddock *et al.*, 1989), Nef (Gilmour *et al.*, 1990) and $p24^{gag}$ (Gilmour *et al.*,

1989), MVV p25^{gag} (Reyburn *et al.*, 1992), ovine tumour necrosis factor α (TNF-α) (Green *et al.*, 1993) and ovine interleukin-1 (IL-1) (Fiskerstrand *et al.*, 1993). Typical yields of fusion protein achieved are in the range 2-5mg/L yeast culture. Exceptionally, yields as high as 50-60mg/L have been reported (Reyburn *et al.*, 1992). Several of the recombinant proteins have been shown to possess biological activity, including HIV-1 Tat (Braddock *et al.*, 1989) and ovine TNF-α (Green *et al.*, 1993). It should be noted that when this system was being developed for Rev expression it was being successfully used in the laboratory for the production of various MVV and ovine proteins.

3.2.1 Generation of Recombinant Ty:Rev Plasmids

A *rev* gene was derived by PCR from cDNA prepared from ovine choroid plexus cells infected with the British isolate of MVV, EV1 (Sargan *et al.*, 1991). The product was cloned into pTZ19R, to produce the plasmid pRev5, and sequenced. pRev5 was used as a template for further PCR using primers incorporating BamHI sites and a sequence corresponding to the recognition site for the protease Factor Xa was also incorporated into the 5' primer. The sequence of these two primers was as follows:



The PCR product was subcloned into the expression vectors pMA5620 and pOGS40 by digestion with BamHI (to yield a 520bp fragment) and subsequent ligation. The products were named pRev.A and pRev.O with reference to the parental vectors, pMA5620 and pOGS40 respectively. This work was carried out by Drs D. Roy and D. Sargan and Mr. I. Bennet.

To validate sub-cloning, the vectors were sequenced across the *tya/rev* boundary. The primer 098G (sequence CAG GAG AAA TCC GAG TG: obtained from C. Cousens),

complementary to a region ~50bp upstream of the cloning site within *tya* was used for double stranded sequencing. Results confirmed the correct subcloning of *rev*.

3.2.2 Sequence Characteristics of the Cloned EV1 Rev Protein

The nucleotide and predicted amino acid sequences of the *rev* insert are illustrated by figure 3.2a. It should be noted that a stop codon at position 148 prevents the translation of 15 carboxy-terminal residues present in the Rev protein of the 1514 strain (Mazarin *et al.*, 1988). The predicted molecular weight of EV1 Rev is 19.5kDa. There are two differences from the EV1 genomic sequence published by Sargan *et al.* (1991). These are T to C at position 32, and A to G at position 117. Only the former substitution results in an amino acid change, replacing a valine residue with an alanine. This does not affect either of the regions with significant homology to the functional domains of the HIV/SIV Rev proteins. These regions are shown in detail in figure 3.2b. The putative basic domain of EV1 Rev (residues 69-90) contains 12/22 arginine and lysine residues and has a central region consisting of 3/4 hydrophobic residues (WFKW). The putative activation domain (residues 104-113) has a significant degree of amino acid identity with HIV-1 Rev, with the presence and distribution of three leucine residues conserved. Overall, the sequence identity between these two proteins is low however (18.1%). The protein has a highly basic character, 27/147 residues are arginine or lysine (18.4%). The predicted isoelectric point (using the UWGCG programme 'Isoelectric') is 10.46. A computer prediction of the secondary structure of Rev is shown in Figure 3.3. The main structural features include predicted α helix between residues 78-90 and 104-119, which includes part or all of both putative functional motifs; and ares of β pleated sheet within the amino terminal regions, at residues 24-33 and 43-57. There are no significant regions of hydrophobicity.

3.3 Transformation of Yeast with Recombinant Plasmids

Cells of the *Saccharomyces cerevisiae* strain BJ2168 were transformed by the lithium acetate based protocol (section 2.12.3) with the plasmids pMA5620 and pOGS40 and with the two recombinant vectors containing the *rev* gene insert (pRev.A and pRev.O). Several DNA concentrations were used, the most successful outcomes were associated with the use

Figure 3.2: Nucleotide and Predicted Amino Acid Sequence of pRev5 Inser

A The nucleotide sequence of the pRev5 insert. The 500bp insert does not show th terminal regions incorporated by PCR, which contain restriction endonuclease and protease cleavage sites (see text 3.2). The two base variations from the published nucleotid sequence of EV1 (Sargan *et al.*, 1991) are highlighted in *italics*. The predicted amino aci sequence obtained by translation of this insert is shown using the three letter code. Th boundary between residues encoded by the first and second exons of the *rev* mRNA i shown (EB).

B Sequence comparison between the amino acid sequence of the EV1 and HIV-1 Re proteins. Comparison made using the UWGCG (ver. 8) programme 'Bestfit'. Identical amin acids are marked by a dash, dots indicate similarity of residue. Regions of homology are limite to the two known functional domains of HIV-1 Rev: the basic (RNA binding and nuclea localisation) and activation domains. These HIV-1 basic domain and the EV1 Rev homolgou region are underlined: the activation domain is shown in *italics*.

A


ATGGCAAGCACAAAGAGCAAGCCAAGCAGAGCAACCTGGACAGACATGGAGCCACCGCAA
TACCGTTCGTGTTTCTCGTTCGGTTCGTCTCGTTGGACCTGTCTGTACCTCGGTGGCGTT
MetAlaSerThrLysSerLysProSerArgAlaThrTrpThrAspMetGluProProGln
AAGGAAAAATGGGGTCAAGTAGTACAAGAGTTGGTGACAAGACAACAAAACGAAGAGAGG
TTCCTTTTTACCCCAGTTCATCATGTTCTCAACCACTGTTCTGTTGTTTTGCTTCTCTCC
LysGluLysTrpGlyGlnValValGlnGluLeuValThrArgGlnGlnAsnGluGluArg
EB
CAGGGATTGGTAACAGGCCTACAAGCAGATTCAACAGATCAGATATACACAGGTAACAGT
GTCCCTAACCATTGTCCGGATGTTCGTCTAAGTTGTCTAGTCTATATGTGTCCATTGTCA
GlnGlyLeuValThrGlyLeuGlnAlaAspSerThrAspGlnIleTyrThrGlyAsnSer
GGTGATAGAAGAACCAGTGGACCTCGAGGAAAAACCAGGAGAAGAAAAGGATGGTTTAAA
CCACTATCTTCTTGGTCACCTGGAGCTCCTTTTTGGTCCTCTTCTTTTCCTACCAAATTT
GlyAspArgArgThrSerGlyProArgGlyLysThrArgArgArgLysGlyTrpPheLys
TGGCTTCGAAGACTTAAGGCAAGAGAAAAGAACATCCCGGCGCACTTTTACCCAGATATG
ACCGAAGCTTCTGAATTCCGTTCTCTTTTCTTGTAGGGCCGCGTGAAAATGGGTCTATAC
TrpLeuArgArgLeuLysAlaArgGluLysAsnIleProAlaHisPheTyrProAspMet
GAGAGCAACGTGGCAGGCCTGGAAAAACTCACCTTGGAAGAAAAGTTGGAGGAGAAGCCT
CTCTCGTTGCACCGTCCGGACCTTTTTGAGTGGAACCTTCTTTTCAACCTCCTCTTCGGA
GluSerAsnValAlaGlyLeuGluLysLeuThrLeuGluGluLysLeuGluGluLysPro
ATATATGAGTCTACTACCTCTACTGGCGGTACATCAGTGGCTGGAAGAGACTGGATGGAT
TATATACTCAGATGATGGAGATGACCGCCATGTAGTCACCGACCTTCTCTGACCTACCTA
IleTyrGluSerThrThrSerThrGlyGlyThrSerValAlaGlyArgAspTrpMetAsp
TGGCGGGAATCAGCTCAAAAATAAAAAAGAAAGGGTGGACTGTCAGGGCAGAGAACAGAT
ACCGCCCTTAGTCGAGTTTTTATTTTTTCTTTCCCACCTGACAGTCCCGTCTCTTGTCTA
TrpArgGluSerAlaGlnLysEndLysArgLysGlyGlyLeuSerGlyGlnArgThrAsp
GCCCATGATTGAGAATGACT
CGGGTACTAACTCTTACTGA
AlaHisAspEnd

B

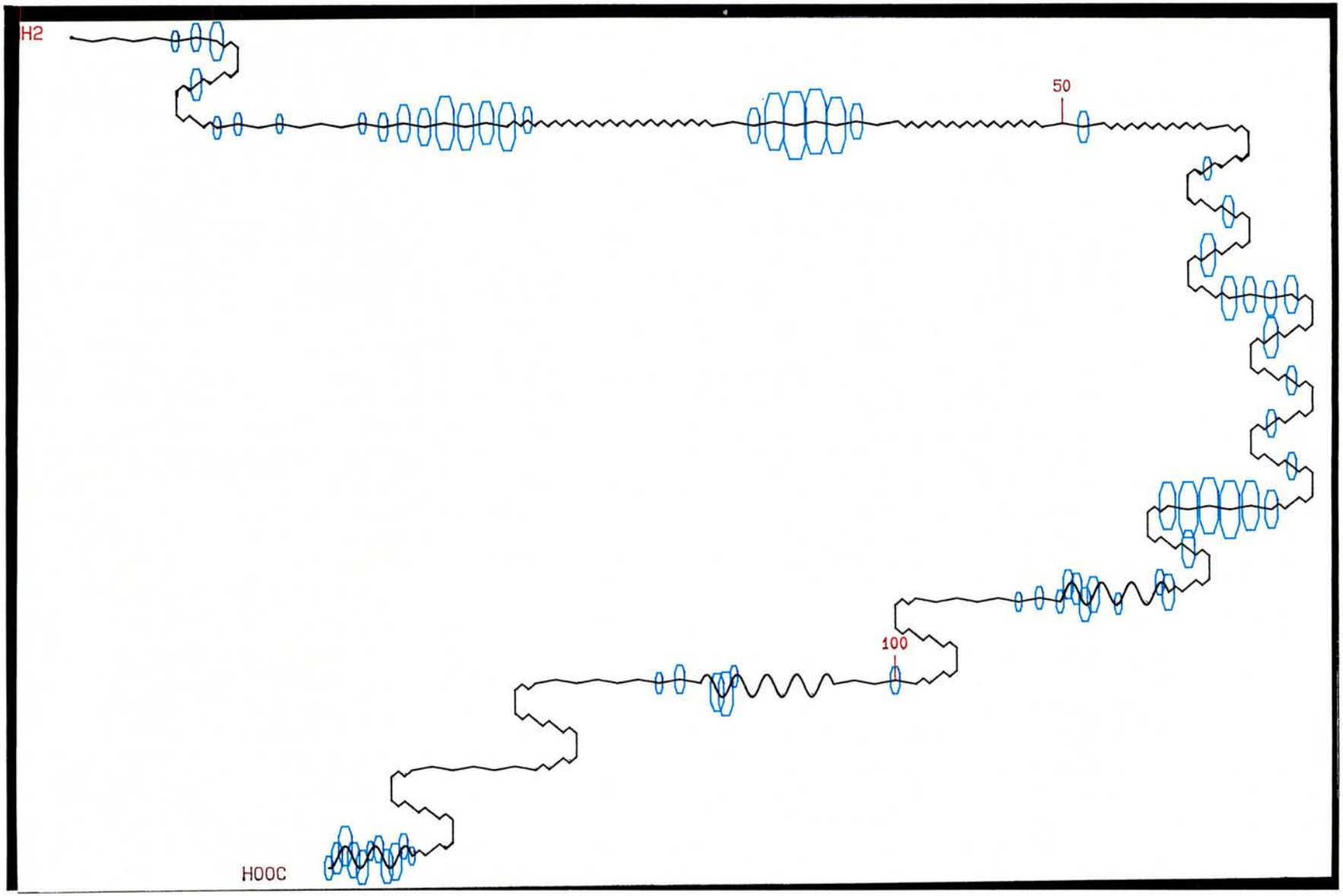
```
  1                                   MAGRSGDSDEDLLKAVRL..IKFLYQSNPPPNPE 32
                                      :. . .:. ::|:.::.   .. :| :|......
  1 MASTKSKPSRATWTDMEPPQKEKWGQVVQELVTRQQNEERQGLVTGLQADSTDQIYTGNSGDRRT 65

        BASIC DOMAIN                          ACTIVATION DOMAIN
 33 GTRQARRNRRRRWRERQRQIHSISERILSTYLGRSAEPVPLQLPPLERLTLD..... 84
    :...:: .||: | .: |.:.. ...| . :..  .. |    :.||:|||:
 66 SGPRGKTRRRKGWFKWLRRLKAREKNIPAHFYPDMESNV....AGLEKLTLEEKLEEKPI 121

 85 CNEDCGTSGTQGVGSPQILVESPTILESGAKE  116   HIV-1:HXB3
    ::...:|:|| ..|.     :. .  ||:.|
122 YESTTSTGGTSVAGR.....DWMDWRESAQK   147   MVV: EV1
```

Figure 3.3: Predicted Higher Order Structure of the EV1 Rev Protein

The secondary structure of EV1 Rev was predicted using the UWGCG programmes 'Ppetide structure' and 'Plotstructure'. Alpha helices are shown as sine waves, beta-pleated sheets are shown in narrow zig-zag from. Blue denotes regions of significant hydrophilicity there are no regions of predicted significant hydrophobicity.


H2
50
100
HOOC

of ~5μg vector and 20μg salmon sperm DNA as carrier. Transformants were selected by leucine independent growth, ie by growth in regeneration agar containing tryptophan and uracil, but not leucine. The various combinations of transfecting plasmids and the outcome of transformation are summarised in table 3.1. Five colonies from each transformation were selected, and re-streaked onto fresh plates. The TRev.A and TRev.O plates were photographed after 2 and 3 days incubation, and are illustrated by figure 3.4. It is clear from both table 3.1 and figure 3.4 that cells transformed with pRev.A display a lag in growth compared to those transformed with pRev.O. The nature of the promoter directing fusion protein expression is the only difference between these transformants: hence, it must be responsible for the observed difference in growth. Therefore, constitutive expression of the

Table 3.1: Transformation of Yeast with the Ty:VLP Vectors.

Cells	Transfected plasmids	No. colonies /plate	Time to colony visibility	Selected colony
T.A	pMA5620	200	2-3 days	8f
T.O	pOGS40	200	2-3 days	6b
TRev.A	pRev.A	10-15	12-14 days	2a
TRev.O	pRev.O	100	4-5 days	5c
TRev.U	pRev.O pUG41S	15	5-6 days	5c.1

fusion protein in the pRev.A transformants must be detrimental to cell growth. Although it is possible that high level expression of the fusion protein acts as a physiological restraint on cell growth, the lack of such an effect on cells constitutively expressing p1 alone strongly suggests that p1:Rev exerts a toxic effect on the host cell. The slightly reduced growth of cells transformed with pRev.O compared to those transformed with pMA5620 further supports this hypothesis; furthermore, this suggests that the PAL promoter is not transcriptionally silent in the absence of the inducing agent. Transformations were carried out

Figure 3.4: Growth of Ty:Rev Transformants in Solid Medium

Five colonies transformed with the Ty-VLP vectors containing the *rev* gene insert were chosen, and re-streaked onto Sc-glc agar plates. The growth of two of these transformants 2e (TRev.A) and 5e (TRev.O) is shown after 48 (**A**) and 72 (**B**) hours incubation.

A

B

with the help of Dr. D. Roy.

3.3.1 Transformation of TRev.O with the Helper Plasmid pUG41S

Co-transformation of cells with both a pOGS40-derived plasmid and pUG41S should lead to enhanced expression from the PAL promoter where the regulatory protein pGAL4 is limiting. TRev.O cells were transformed with 5-10μg of pUG41S according to the above parameters. Transformants were selected on the basis of leucine and uracil independent growth on plates containing Sc-glc agar and tryptophan. As the presence of pUG41S should correlate with increased expression of fusion protein, even in the absence of IPTG, the delay in visible colony formation (5/6 days compared to 4/5 days for pRev.O alone, table 3.1) is further evidence to suggest a toxic effect of p1:Rev expression. Doubly transformed cells were denoted TRev.U. One colony, TRev.U.5c.1, was chosen for future experiments.

3.4 Validation of Fusion Protein Expression by Transformants

Selected transformants were screened for expression of fusion protein by generation of small scale (50ml) cultures, with induction with galactose where appropriate. Cells were harvested after 48 hours growth, and crude extracts prepared by lysing cells with glass beads. Protein production was analysed by SDS-PAGE. Gels were stained with Coomassie blue, or electroblotted onto nitrocellulose and probed with a polyclonal anti-p1 antiserum (a kind gift of British Biotechnology Ltd.). A blot positive band with apparent molecular weight in the range 55-58kDa is present in the p1 extracts (figure 3.5a,b). Although this is considerably larger than the expected weight of 42kDa, reduced mobility of p1 in this gel system has previously been observed (Adams *et al*, 1987a). By comparison with the p1 extract, the tracks corresponding to the Ty:Rev transformants demonstrate loss of the p1 band and its replacement with a band with size in the range 74-76kDa. The expected molecular weight of p1:Rev fusion protein is 61.5kDa (42kDa p1 + 19.5kDa Rev), the altered mobility of the p1 moiety is presumably responsible for the observed discrepancy. There may also be an anomaly due to the high basic content of Rev, a feature which is associated with reduced mobility in SDS-PAGE (Gabriel and Wong, 1969). This band was therefore assigned to the expressed fusion protein, p1:Rev. A sample of purified p1:$p25^{gag}$ included as a control, has

Figure 3.5: Validation of Fusion Protein Production by Ty Transformants.

Cell extracts were analysed by electrophoresis through 10% polyacrylamide gels. Tota protein content of extracts was equalised by use of the Biorad assay. Gels were stained wit Coomassie blue (**C**,**D**) or Western blotted and probed with anti-p1 antiserum (1/1000 dilutior (**A**,**B**).

Extracts: BJ: Untransformed BJ2168 cells

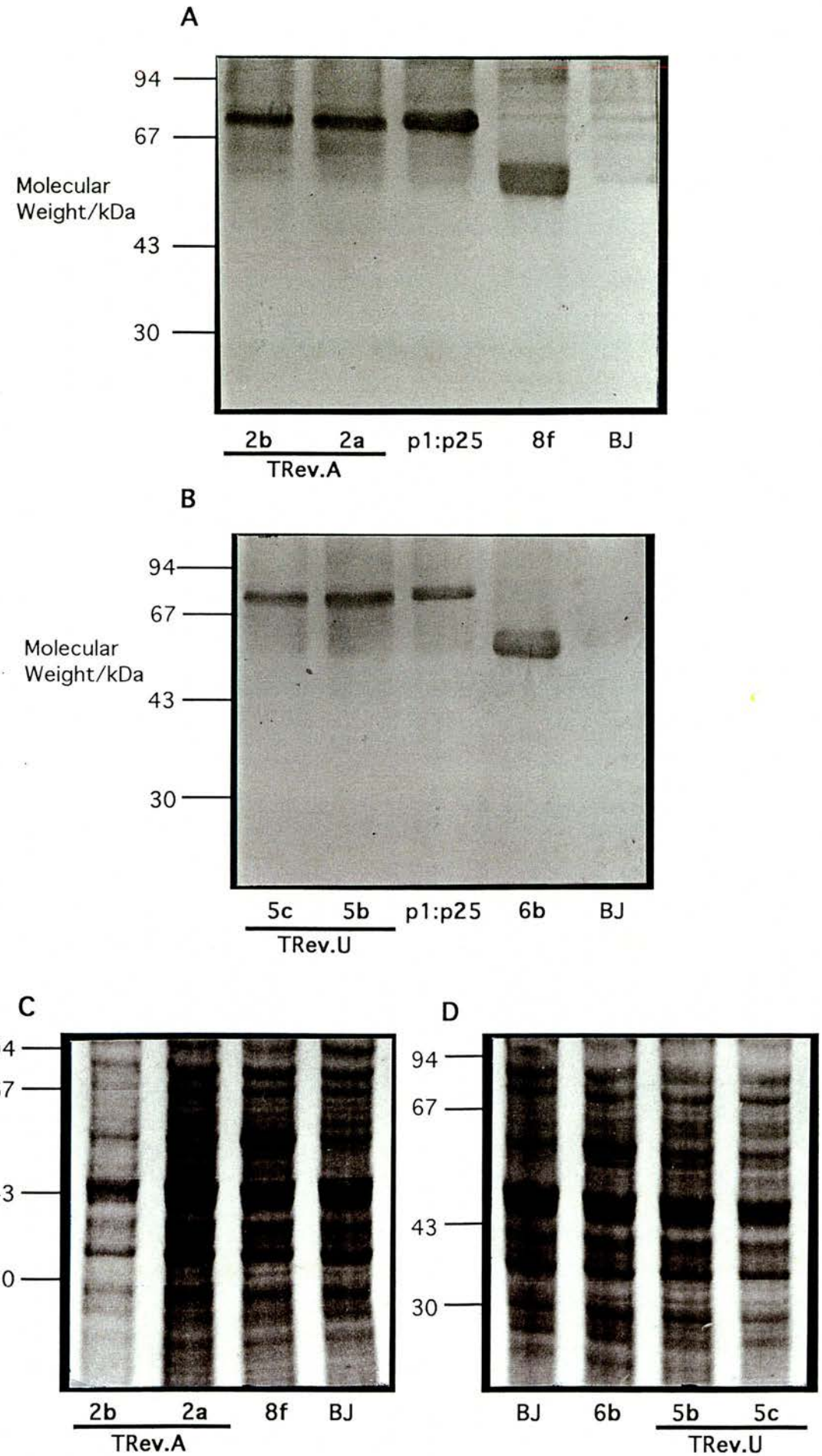
T.A: 8f

T.O: 6b

TRev.A: 2a, 2b

TRev.U: 5b, 5c

p1:p25^{gag} obtained from a purified preparation (a kind gift of Mr. Hugh Reyburn, Departmer of Veterinary Pathology, University of Edinburgh).


A
94
67
Molecular
Weight/kDa
43
30
2b
2a
p1:p25
8f
BJ
TRev.A
B
94
67
Molecular
Weight/kDa
43
30
5c
5b
p1:p25
6b
BJ
TRev.U
C
94
67
43
30
2b
2a
8f
BJ
TRev.A
D
94
67
43
30
BJ
6b
5b
5c
TRev.U

slightly reduced mobility when compared to p1:Rev. No blot positive material was present in extracts from untransformed BJ2168 cells, suggesting that endogenous Ty elements were not expressed under these conditions.

There is little observed difference in terms of p1:Rev band intensity between TRev.A (A) and TRev.U (B) cells, nor between TRev.U and TRev.O (data not shown). It should be noted that in order to equate the amount of protein in each gel track, approximately 4µl of p1:Rev extract was loaded, compared with 1µl of p1 extract. This suggests that the p1:Rev expressing transformants might be low yielding. Examination of Coomassie stained gels (figure 3.5c,d) failed to identify a band corresponding to p1:Rev in the whole cell extracts. By contrast, a band of enhanced intensity (arrowed), running at ~55kDa and thus corresponding to p1, is clearly visible in the T.A and T.U tracks.This indicates that the yield of p1:Rev is less than that of native p1. The toxic effect of the *rev* insert identified during transformation (3.3 above) is a likely mechanism for reduced yield. It was decided that the TRev.U colonies would be used for further study, in the belief that the inducible nature of p1:Rev expression would potentially allow improved growth and yield compared to the constitutively expressing TRev.A cells.

3.5 Large Scale Production and Purification of p1:Rev

Large scale protein purification was performed on extracts from four-litre cultures of induced TRev.U cells. Culture conditions and purification protocol were based on the methods of Adams *et al.* (1991), and are as described in section 2.12.4.2. As a control, native p1 protein was purified from T.A cells (transformed with the constitutive expression vector pMA5620). Sucrose gradient fractions were analysed by resolution of protein by SDS-PAGE. The Coomassie stained profiles of both p1:Rev (Figure 3.6a) and p1 (3.6b) gradients are presented. Much of the contaminating yeast protein accumulates in the upper section of the gradients, with the particulate VLPs sedimenting in lower sections. p1 protein concentrates in fractions 5-10 (where fraction 1 is the top of the gradient and fraction 19 is the bottom), where it is the predominant protein. These fractions were pooled, dialysed against TEN buffer, and subjected to a further separation on a 15-45% sucrose gradient. By Coomassie staining, this results in an essentially homogeneous preparation (figure 3.6d) with a total yield of approximately 5mg/L yeast culture. By contrast, there is no discernible band due to p1:Rev

Figure 3.6: Purification of Ty-VLPs by Density Centrifugation

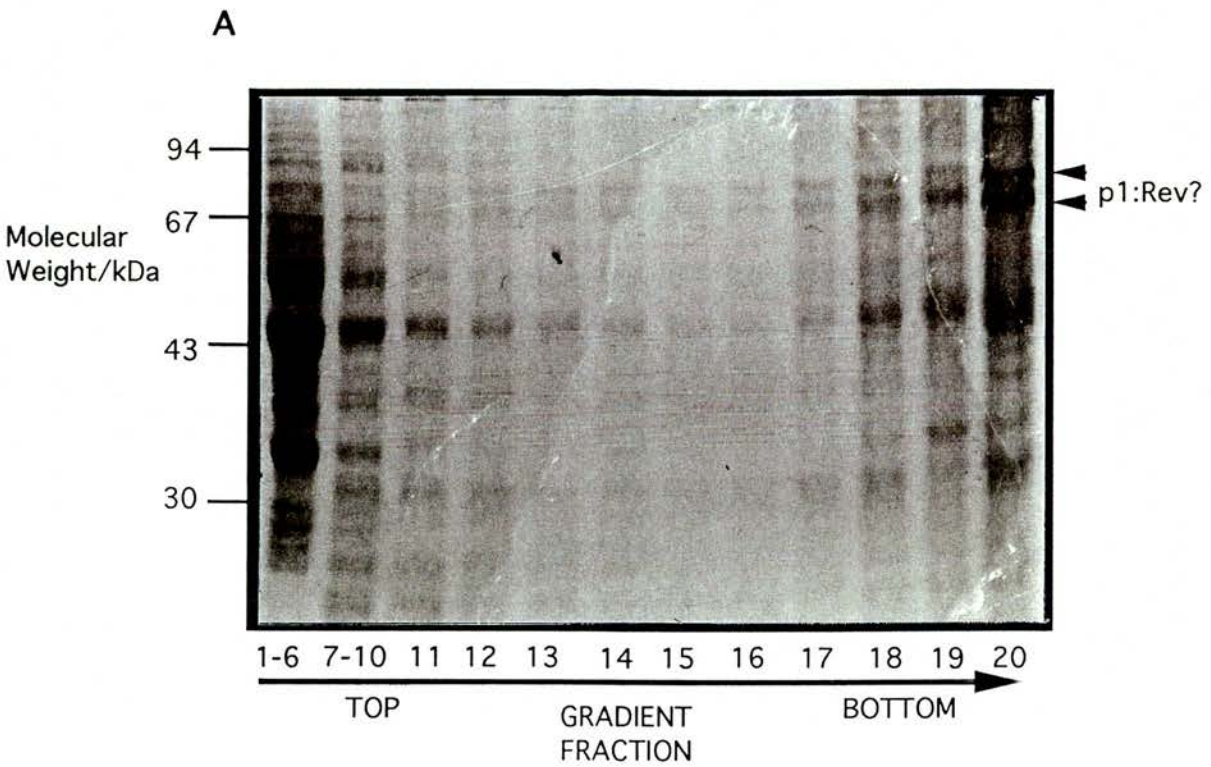
A A 4l culture of TRev.U cells were induced to express fusion protein by incubation i Sc-glc-gal medium for 24 hours as described (2.12.5). Cell extracts were prepared, an cellular debris removed (2.12.6). Purification of recombinant VLPs was achieved by densit centrifugation through a 15-45% sucrose gradient (2.12.7). Fractions (2ml) were collecte from the gradient, and samples (10µl) resolved by SDS-PAGE through a 10% polyacrylamid gel. Visualisation is by staining with Coomassie blue. Gradient fractions are numbered from (top) to 19 (bottom). Fraction 20 contains material derived from a 2ml 60% sucrose cushio underlaying the gradient.

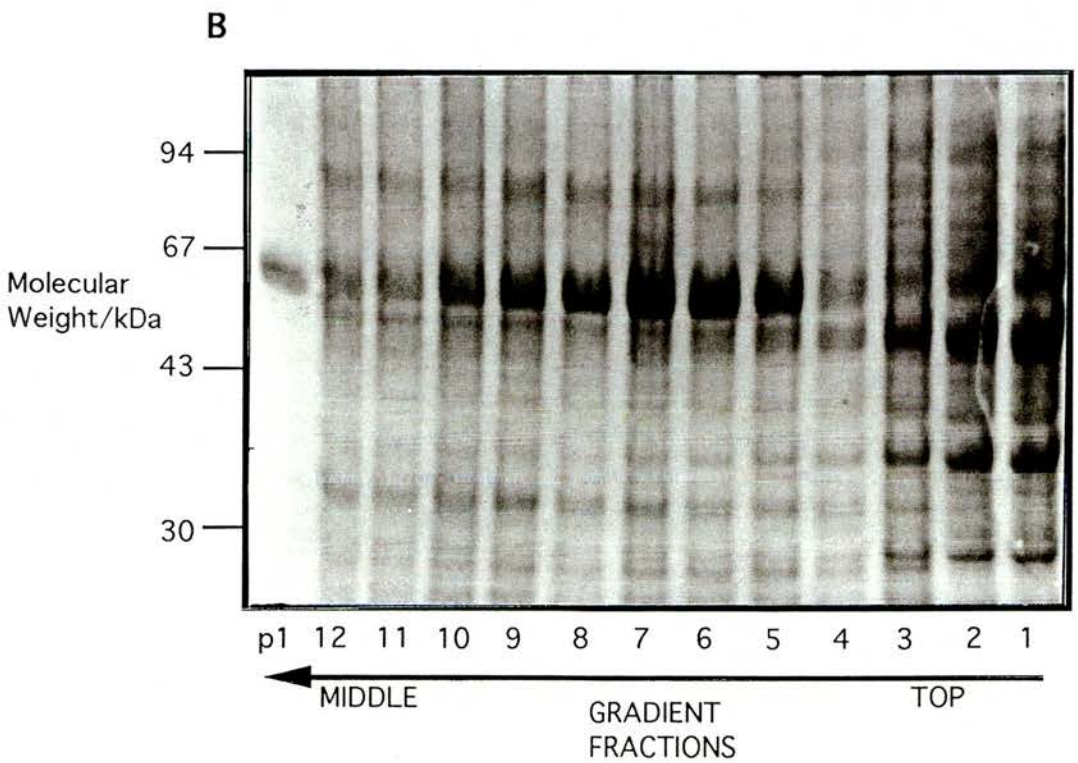
B A 4l culture of T.A cells was incubated for 24hrs in Sc-glc medium, and treated a above (A). A sample of pre-purified p1 is included as an internal marker.

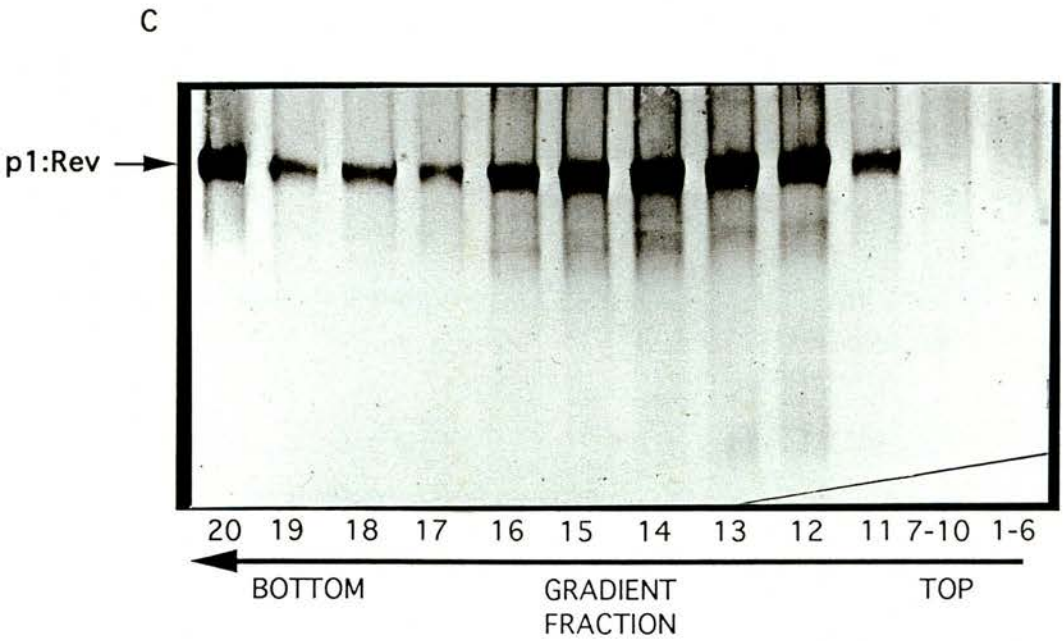
C A similar gel to (A) above was blotted onto nitrocellulose and probed with anti-p antiserum (1/1000 dilution).

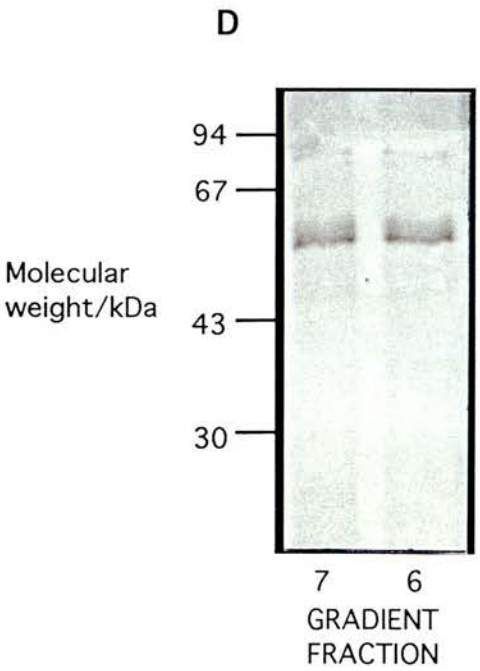
D Fractions from the T.A sucrose gradient containing peak concentrations of p1 (5-1(were pooled and subjected to a second round of density centrifugation. Fractions wer collected and analysed as above (A). Figure shows two fractions containing purified p1.

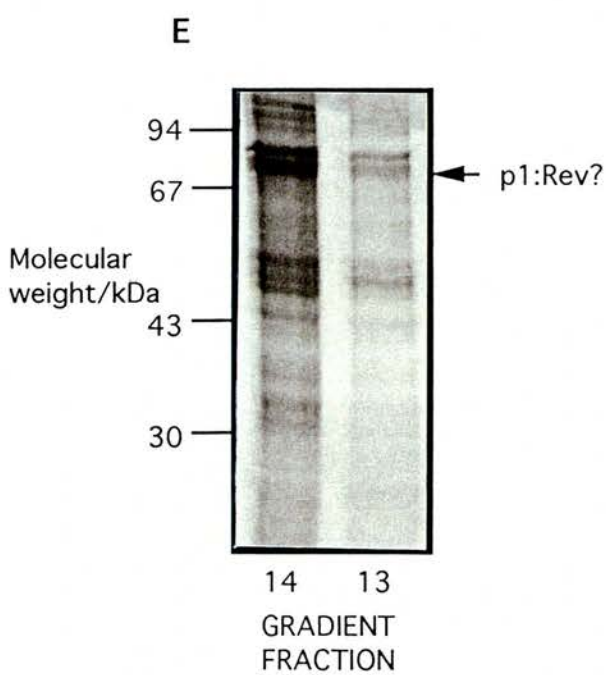
E Fractions from the TRev.U sucrose gradient containing peak concentrations p1:Rev (12-16: identified by Western blotting) were pooled and subjected to a second rour of density centrifugation. Fractions were collected and analysed as above (A). Figure show two fractions containing putative purified p1:Rev. The candidate band corresponding p1:Rev is indicated by an arrow.


A
94
67
43
30
Molecular Weight/kDa
p1:Rev?
1-6 7-10 11 12 13 14 15 16 17 18 19 20
TOP
GRADIENT FRACTION
BOTTOM


B
94
67
43
30
Molecular Weight/kDa
p1 12 11 10 9 8 7 6 5 4 3 2 1
MIDDLE
GRADIENT FRACTIONS
TOP


C
p1:Rev
20 19 18 17 16 15 14 13 12 11 7-10 1-6
BOTTOM
GRADIENT FRACTION
TOP


D
94
67
43
30
Molecular weight/kDa
7 6
GRADIENT FRACTION


E
94
67
43
30
Molecular weight/kDa
p1:Rev?
14 13
GRADIENT FRACTION

visible by Coomassie staining. Although two possible candidates (figure 3.6a: arrows) are present in the expected location, a western blot of the p1:Rev gradient (Figure 3.6c) demonstrates accumulation of the protein in fractions 12-16, a distribution which does not correlate with that of these bands. A proportion of p1:Rev falls into the 60% cushion. The fact that this concentration of p1:Rev occurs without a parallel increase in visibility by Coomassie staining leads to the conclusion that p1:Rev is produced at a much lower level than native p1 protein. The peak fractions for p1:Rev content were pooled, sucrose removed by sephadex chromatography (section 3.9.3.1) or by dialysis, and subjected to a second round of centrifugation through a 15-45% sucrose gradient. The VLPs were found to behave in a similar manner to the first sedimentation. Blot positive fractions (14 and 15) were analysed by Coomassie staining (Figure 3.6e). These samples appear to demonstrate concentration of a band of approximately 72-75kDa which is not present in blot negative fractions (figure 3.6e arrow). This band is the faster migrating of two bands found in a similar position to those seen after the first sedimentation (3.6a) and may represent resolution of these proteins. This was therefore assigned as a candidate band for p1:Rev. However, further purification would be required for an unequivocal demonstration of the identity of this band. The purity of this protein as estimated from the Coomassie staining profile was ~10%. The overall protein concentration in fractions 14 and 15 was 1.2mg/ml, giving an approximate total yield of p1:Rev of 480µg (120µg/L yeast culture). The second gradient fraction 14 (F14) material was used as the main source of p1:Rev for further studies.

Purification of VLPs relies on a relative rather than absolute separation of yeast proteins, such that a high yield of recombinant protein is essential to generate a highly pure preparation. One possible explanation for the low yield of p1:Rev is instability of the fusion protein. Many yeast proteases are located within the vacuole, and can attack the expressed protein on release by cell lysis. However, the strain used here is deficient in several known proteases, and is associated with stability of several other heterologous proteins. Particle formation is thought to promote fusion protein stability (Gilmour *et al.*, 1989). Moreover, there is no evidence for the production of intermediates by proteolysis by western blotting detection. Although instability cannot be ruled out, the previously observed inhibitory effect of p1:Rev expression on the growth of yeast cultures suggests that a toxic mechanism for low yield is more likely. To investigate this possibility, a time course study of expression was performed, and the effects of p1:Rev on yeast morphology examined by microscopy.

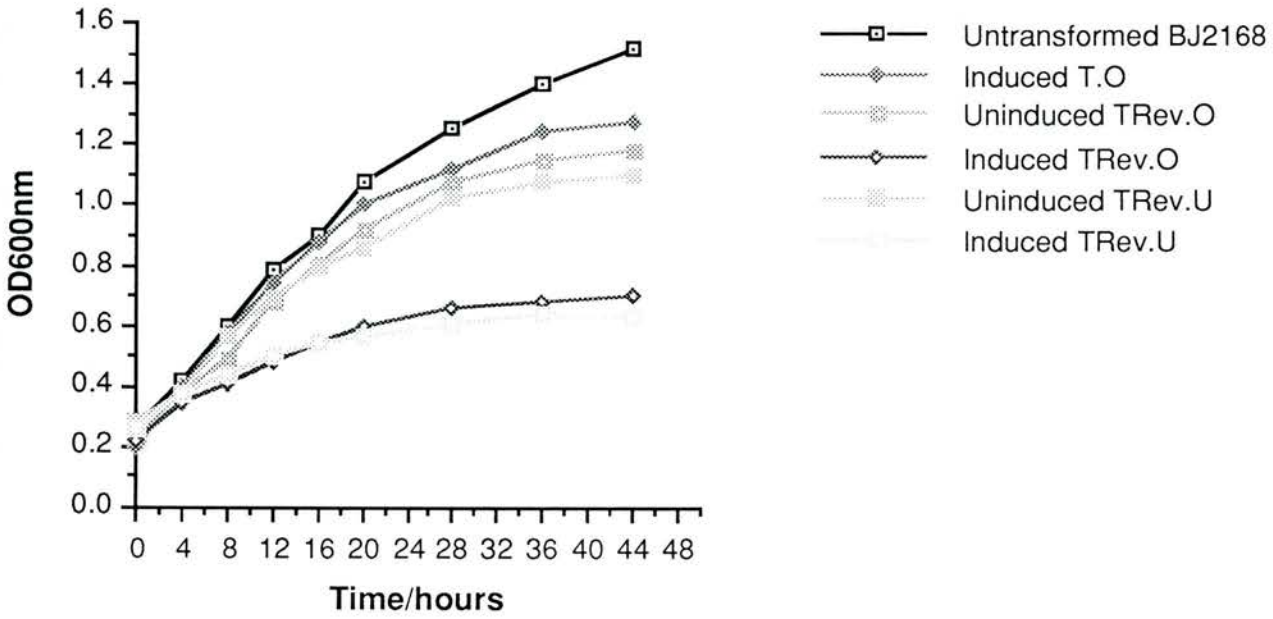
3.6 Time Course of Fusion Protein Expression

The growth characteristics of both induced and uninduced pRev.O transformants were investigated in order to analyse the observed retardation of growth of cells containing the *rev* insert. Transformants were grown in Sc-glc/trp until an OD_{600nm} of 0.2-0.3 had been reached. The cells were then pelleted and resuspended in pre-warmed Sc-glc/trp or Sc-glc-gal/trp where appropriate, followed by continued incubation. Culture turbidity was recorded at four hour intervals for 20 hours, and subsequently at eight hour intervals until 44 hours. Protein expression was monitored at regular intervals by removal of 50ml aliquots of culture for the preparation of crude cellular extracts. This work was performed in conjunction with Mr. J. Warden.

Figure 3.7a demonstrates the growth profiles obtained in this experiment. Untransformed cells grew rapidly, although growth rate was not optimal due to the poor uptake of galactose by these GAL2 mutants. It is clear that the growth of cultures expressing p1:Rev is dramatically inhibited. Reduction in growth is evident from as early as eight hours, and growth effectively has ceased when the cultures attained a density of ~$2x10^7$ cells/ml. Dilution of an aliquot of stationary cells in fresh, non-galactose containing media did not lead to an increase in culture growth, suggesting that inhibition of growth is irreversible. Growth of cells doubly transformed with pRev.O and pUG41S (TRev.U) would appear to lag slightly behind that of singly transformed cells, although as only a single experiment was performed for each transformant it is impossible to determine the significance of this observation. The growth of TRev.O and TRev.U cultures in non-inducing conditions would appear to be retarded in comparison with untransformed cells, correlating with previous observations of colony formation in solid phase media in the absence of galactose. Cells expressing p1 alone (T.O) demonstrate some reduction in growth in late log phase.

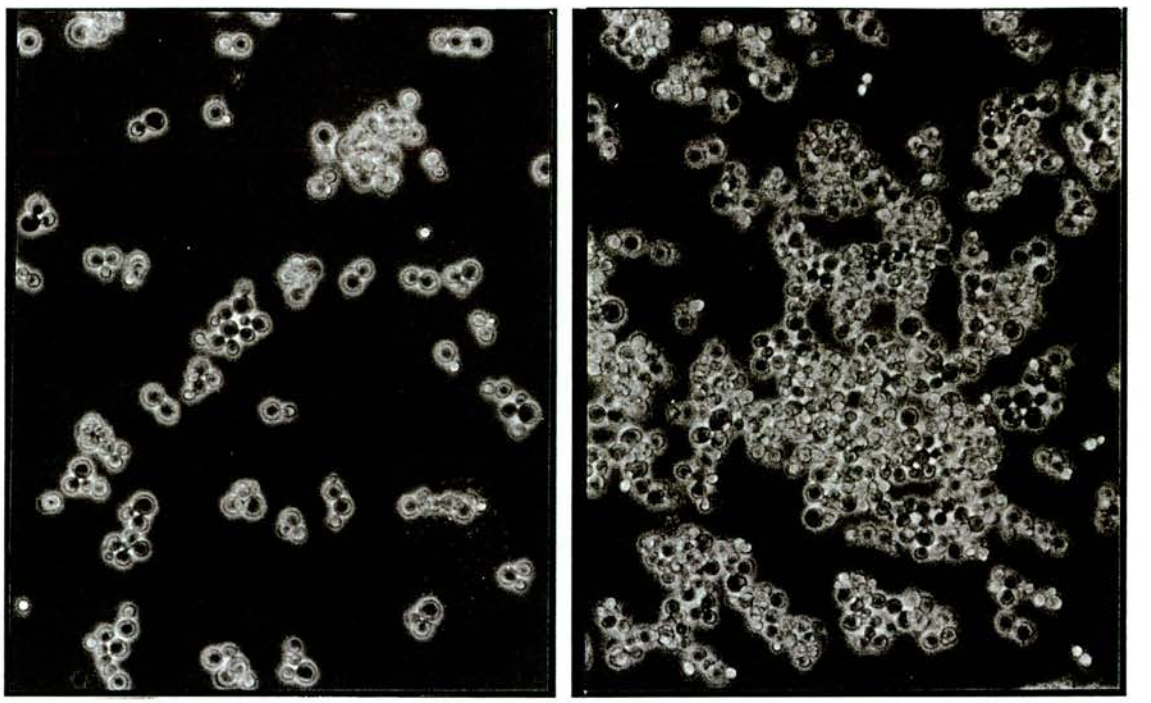
Light microscopic examination of cells at various time points further confirmed the toxic nature of p1:Rev. At 24 hours post-galactose shift, the appearance of untransformed cells was essentially normal (Figure 3.7b.i), with budding cells forming chains characteristic of rapid growth in good conditions. However, at the same time point, TRev.U cells formed large, dense aggregates (Figure 3.7b.ii). This is strongly suggestive of cell death rather than of a stationary phase culture. At this point the overall culture density was approximately half that of

A

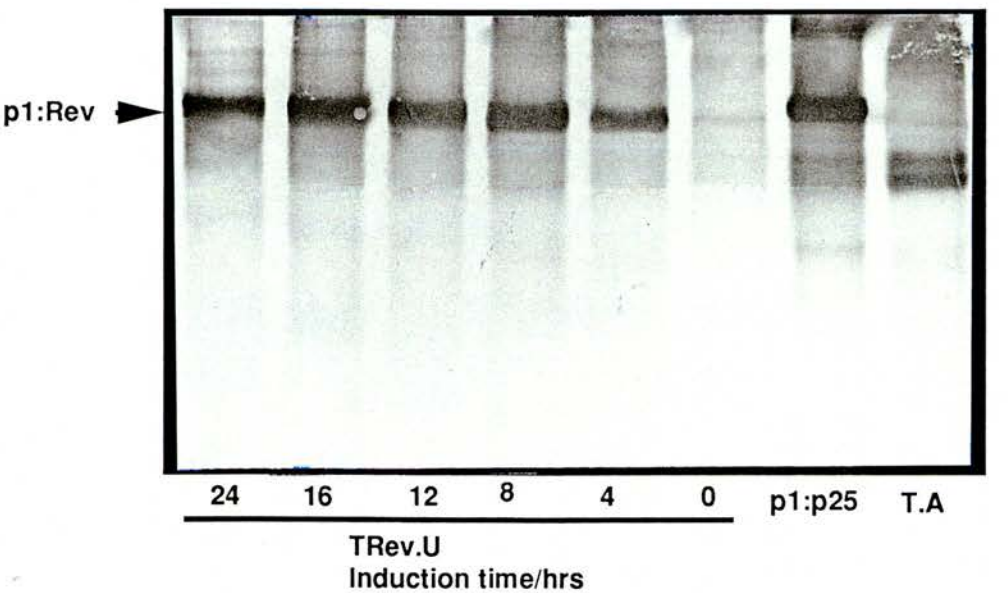

OD600nm
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 4 8 12 16 20 24 28 32 36 40 44 48
Time/hours
Untransformed BJ2168
Induced T.O
Uninduced TRev.O
Induced TRev.O
Uninduced TRev.U
Induced TRev.U

Bi

Bii



C



the untransformed cell culture. Aggregates were clearly visible by microscopic examination as early as eight hours post induction. Clumping of cells also occurred to a lesser extent in uninduced TRev.O and TRev.U cultures at later time points. Cells expressing p1 did not demonstrate clumping and appeared healthy. This suggests that the observed reduction in culture growth was due to the physiological effects of high level expression of p1, rather than to a toxic mechanism.

Protein production at various time points was examined by SDS-PAGE analysis of crude extracts of harvested TRev.U cells. Recombinant protein was detected by probing Western blots with anti-p1 antiserum (Figure 3.7c). p1:Rev was clearly detectable, though at low levels, prior to induction, thus confirming the previous suggestion that the pOGS40 PAL promoter is incompletely repressed in the absence of galactose. After induction it is evident that the amount of fusion protein detected does not alter greatly with time. This might be expected given the observed growth characteristics of induced TRev.U cells. One can hypothesise that cellular expression of p1:Rev is accompanied rapidly by toxic effect, leading to cell death.

In conclusion, the toxic nature of p1:Rev expression has been confirmed. It is not known whether this effect is mediated at the mRNA or protein level. The best strategy for the large scale production of p1:Rev would therefore appear to be the attainment of a high density culture before induction, with the induction period itself kept to a minimum.

3.7 Examination of VLPs and Expressing Cells by Electron Microscopy

Transmission electron microscopic examination of sucrose gradient fractions containing p1:Rev confirmed the production and isolation of VLPs. Recombinant VLPs (Figure 3.8b) possess an electron-light 'shell' surrounding an electron-dense core region. Particles have a largely spherical morphology, although there is a degree of heterogeneity in shape not observed in native VLPs. With particle diameters in the range 30-100nm, p1:Rev VLPs are also larger than native VLPs (20-40nm). Aggregations of recombinant particles were observed throughout the microscope field. Unidentified electron-light material is associated with such aggregates. Although possibly an artefact of the staining process, this phenomenon has not been observed to occur with purified native or other recombinant particles.

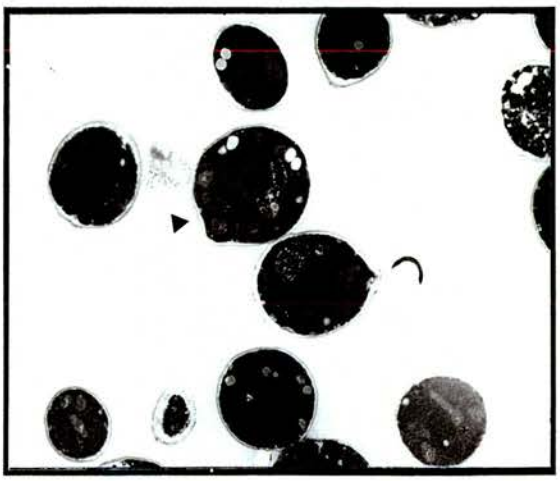
Figure 3.8: Electron Microscopic Examination of Ty-VLP Expression.

Ai-iv Transmission electron micrographic examination of TRev.U yeast cells at variou points post-shift into Sc-glc-gal/trp. Cells were fixed in osmium tetroxide (2.17.2 Representative cross-sections are presented. Arrows denote features discussed in the text Magnification x 3550

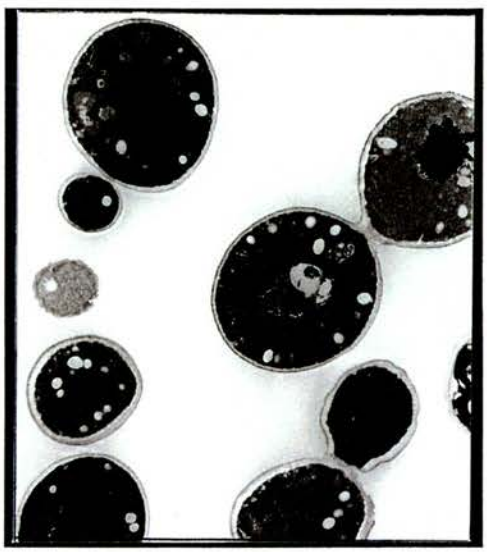
B Isolated Ty-Rev VLPs. 10µl sample of second gradient fraction14 (F14) material fro p1:Rev purification applied to a grid and the sample was negatively stained by fixing in uran nitrate (2.17.1).
Magnification x 110000

C Ty-VLPs within yeast cell. TRev.U cell from 12hrs post-induction. Cell ultrastructur features are identified. Putative Ty-Rev VLPs are denoted by an arrow.
Magnification x 71000

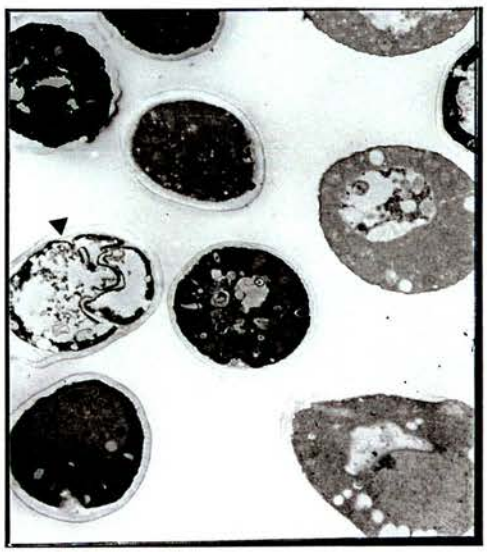
Ai 0 hours



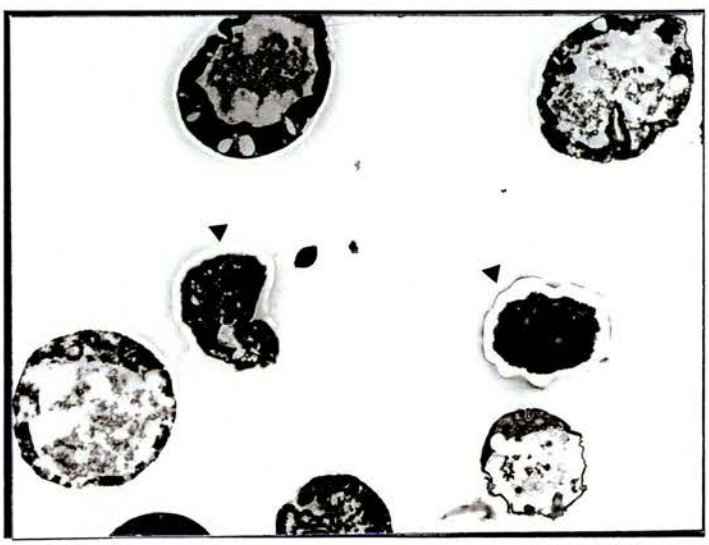
ii 4 hours



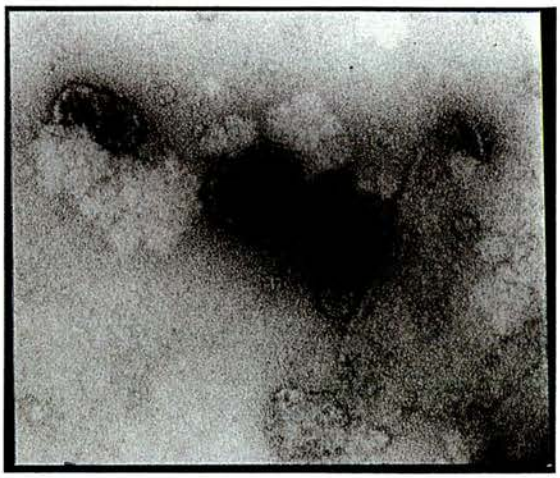
iii 12 hours



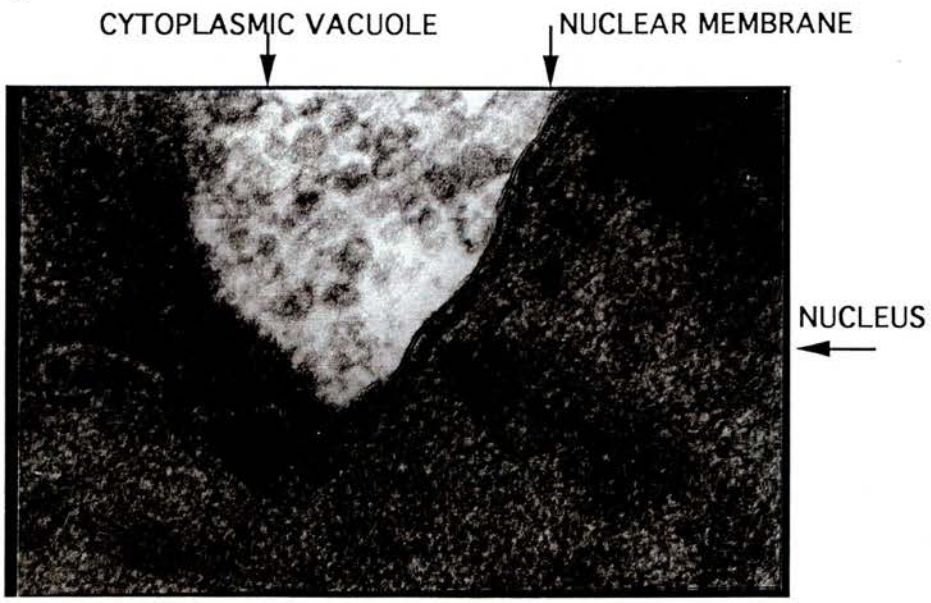
iv 24 hours



B



C



Analysis of cells expressing p1:Rev VLPs at various points of the time course experiment (section 3.6) demonstrated the severe toxic effect of expression of this protein. Prior to induction, the majority of TRev.U cells displayed normal morphology, with evidence of budding forms (Figure 3.8b i, arrows). A small proportion, however, possess enlarged, poorly defined central vacuoles, characteristic of unhealthy cells. The percentage of such cells increases with induction time (3.8b ii-iv). Many cells assume an irregular shape, and display increased diameter: this may be a result of arrested cell division. Vacuolation in severely affected cells proceeds to the stage where the normal cellular ultrastructure has been completely destroyed, with tight packing of cellular components between the vacuole and outer membrane (3.8b iii, arrow). These cells are obviously dead. It is not known whether autolysis of dead cells occurs, although some cells at the 24 hour time point have a condensed appearance suggestive of a post-lysis stage (3.8b iv, arrows). At 24 hours post induction essentially normal cells are rare. In contrast, T.U cells expressing native VLPs retained normal morphology throughout the induction period (data not shown). By examination at a higher magnification, candidate recombinant VLPs were observed at a low frequency as aggregates within the cytoplasm, though not the nucleus, of cells retaining a degree of normal structure (3.8c). There is no equivalent, however, of the densely packed arrays of VLPs found in cells expressing native p1 VLPs (Mellor *et al.*, 1986).

The toxic effect of p1:Rev expression on host yeast cells has therefore been shown to involve dramatic alterations in cell morphology. Such changes are almost certainly irreversible in nature, leading to cell death. p1:Rev induced cell enlargement may explain the slight increase in culture turbidity at 8-20 hours post induction, at a point where viable cell numbers are probably decreasing, rather than increasing.

3.8 Production of Polyclonal Antisera Against Ty-VLPs

The particulate nature of VLPs suggests that they are likely to act as good immunogens. Production of an anti-Rev antiserum would be potentially useful in the further purification of Rev protein, in addition to investigation of other aspects of Rev biology. A fresh anti-p1 antiserum was also prepared. Rabbits were immunised with either 100μg p1 (rabbit R194) or ~200μg of p1:Rev preparation (of which 20-40μg was estimated to be fusion protein) (rabbit R190) derived from second gradient fraction 14, according to the protocol

3.9: Characterisation of Polyclonal Rabbit Antisera Raised Against Ty-VLPs

Polyclonal antisera were obtained by immunisation of rabbits with p1 (R194) c
p1:Rev (R190) containing material as described in the main text. Pre-immune and immur
sera were tested for reactivity with both p1 and recombinant proteins by probing western blo
derived from 10% polyacrylamide gels. Antigens present in each blot are indicated.

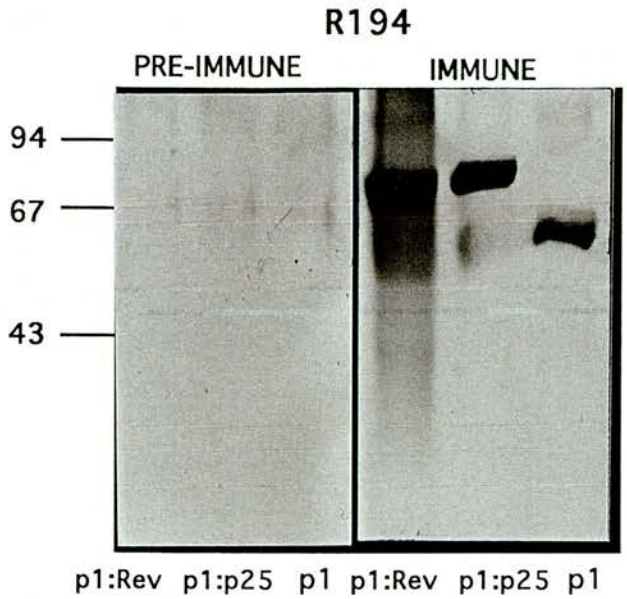
A **R194** anti-p1 (1/1000 dilution)

B **R190** anti-p1:Rev (1/100 dilution)

C R190 antiserum was further characterised by probing extracts of ovine fibroblas
from several time points post-infection with the EV1 strain of MVV (a kind gift from Dr. D Roy
Extracts resolved by electrophoresis through a 10-20% polyacrylamide gradient ge
Antiserum used at 1/100 dilution.

D R190 reactivity with EV1 Rev protein expressed as a fusion to the protein glutathior
S-transferase (GST) in bacteria (chapter 4). A sample of p1:p25^{gag} is included for compariso

A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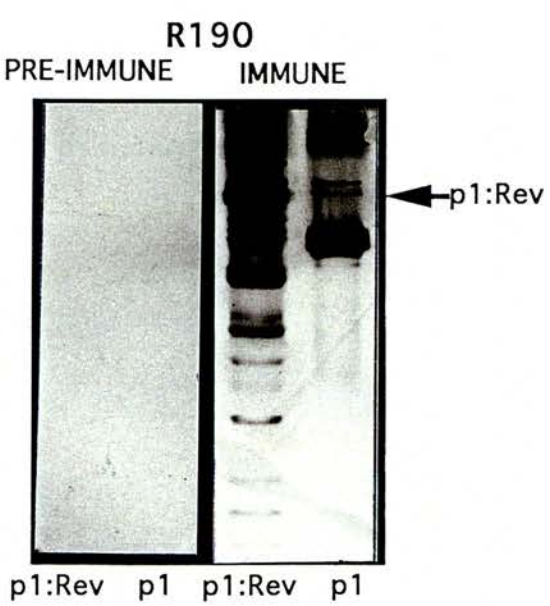
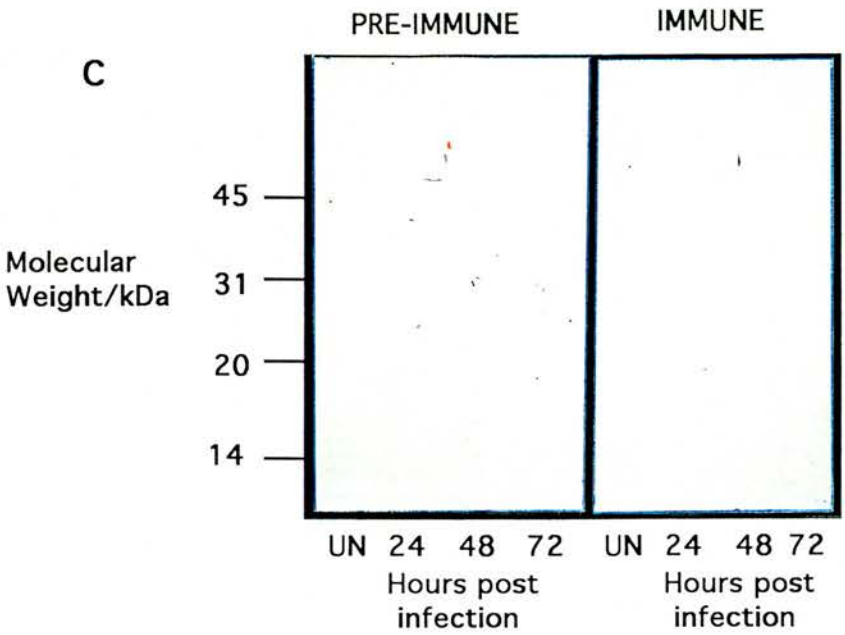
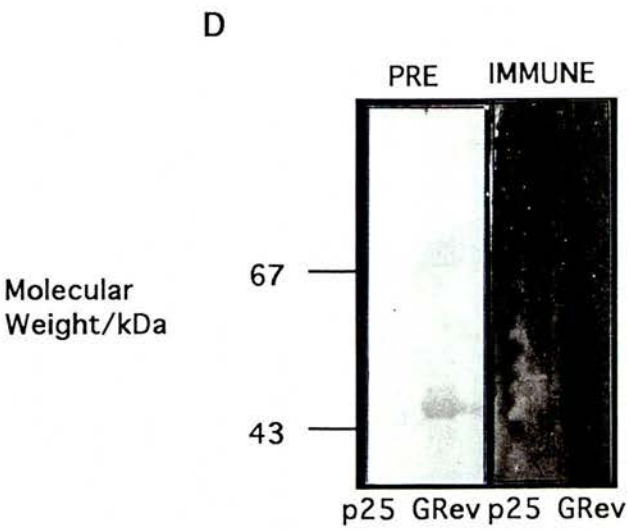
PRE-IMMUNE
IMMUNE
94
67
43
p1:Rev p1:p25 p1 p1:Rev p1:p25 p1

B


R190
PRE-IMMUNE
IMMUNE
p1:Rev
p1:Rev p1 p1:Rev p1


C
PRE-IMMUNE
IMMUNE
Molecular Weight/kDa
45
31
20
14
UN 24 48 72
Hours post infection
UN 24 48 72
Hours post infection


D
PRE
IMMUNE
Molecular Weight/kDa
67
43
p25 GRev p25 GRev

outlined in section 2.23.1. Serum obtained 24 days after primary injection was tested for specificity by western blotting. Antiserum from R194 was found to react with p1 and the p1-derived proteins p1:p25^{gag} and p1:Rev (Figure 3.9a); serum from the pre-immune animal gave no such reactivity. R194 therefore demonstrates the same specificity as the anti-p1 antiserum obtained from BBL. Blots of the p1:Rev fraction 14 material used to immunise R190, and p1 and p1:p25^{gag} were probed with both pre-immune and the resulting antiserum (Figure 3.9b). p1:Rev was detected by the immune serum only. Although a number of other proteins were recognised by this serum, the strongest reactivity was with p1:Rev, as might be expected given the particulate nature of the VLPs. Strong reactivity with p1 was also recorded. Further experiments were performed in order to resolve the reactivity of R190 with p1:Rev into anti-p1 and anti-Rev components. To identify anti-Rev reactivity, R190 was used to probe extracts from fibroblasts infected with EV1 (a kind gift of Dr. D. Roy, Department of Veterinary Pathology, University of Edinburgh). No reactivity was demonstrated (Figure 3.9c), despite the presence of Rev-specific mRNA in these cells (D. Roy, personal communication). R190 antiserum also failed to identify a Rev-specific band on protease cleavage of the fusion protein (see section 3.11). Reactivity with a bacterial Rev fusion protein, GSTRev (chapter 4) was characteristic of both the pre-immune and immune sera (3.9d), suggesting that reactivity may be anti-GST in nature. The reactivity of R190 with p1:Rev would thus appear to be entirely due to recognition of the p1 moiety of the fusion protein, with no evidence for anti-Rev activity. This was surprising, given the proposed advantages of antigen presentation in the form of polyvalent particles (Adams *et al.*, 1987).

3.9 Attempts to Improve Yield and Purity of p1:Rev

Three approaches were taken in an attempt to increase the yield and purity of p1:Rev fusion protein. These were; modifying the growth and expression protocol to moderate the effects of Rev-mediated toxicity; minimising the loss of Ty-Rev VLPs during steps in the purification protocol, and finally, the use of alternative methods of purification.

3.9.1 Culture Conditions and Expression

In order to increase yield of expressed protein by altering culture conditions it would be necessary to boost the number of expressing cells before and/or during induction, or to increase the effectiveness of induction, for example by altering the delivery of the inducing agent. Expression of p1:Rev is accompanied rapidly by cell death, placing a limit to the amount of protein expressed by each cell. Thus, only by increasing the number of cells can the overall yield be increased. Growth of cells transformed with pRev.O in glucose is hampered by the observed 'leakiness' of the PAL promoter, resulting in cell death in the absence of the inducing agent. It was found that the conditions used above resulted in a high proportion of clumped, dead cells, before induction. Several modifications to this protocol were essayed. The most successful of these is described in section 2.12.7.2.2. This involved a gradual increase in culture volume, with dead cells being eliminated at each step. At later stages, 0.2% yeast extract was added. Although this had the result of promoting growth, it may have been at the expense of a degree of loss of the transformed state, due to the presence in yeast extract of the selective agents leucine and uracil. Immediately prior to induction the culture contained an estimated 20% clumped yeast, as determined by light microscopy. This figure was lower than that seen with other protocols. The induction period was set at seven hours, at which point a proportion of viable cells remained.

However, significant increases in the final yield of p1:rev were not apparent despite the improved growth. This suggests that increases in the number of VLPs were insufficient to overcome limiting parameters within the purification process.

3.9.2 Minimising Loss of p1:Rev During Purification

Significant loss of fusion protein was likely to occur at three main stages of the purification protocol. Incomplete lysis of cells would reduce the volume of protein available for purification. Cell lysis was monitored by microscopic examination. Greater than 80% lysis was achieved on each occasion, thus reducing the potential variation between preparations. The proportion of protein lost due to sequestration within intact cells was therefore kept to the minimum possible level.

Pelleting of p1:Rev within cellular and macromolecular debris during pre-sucrose

Figure 3.10: VLP Loss During Purification

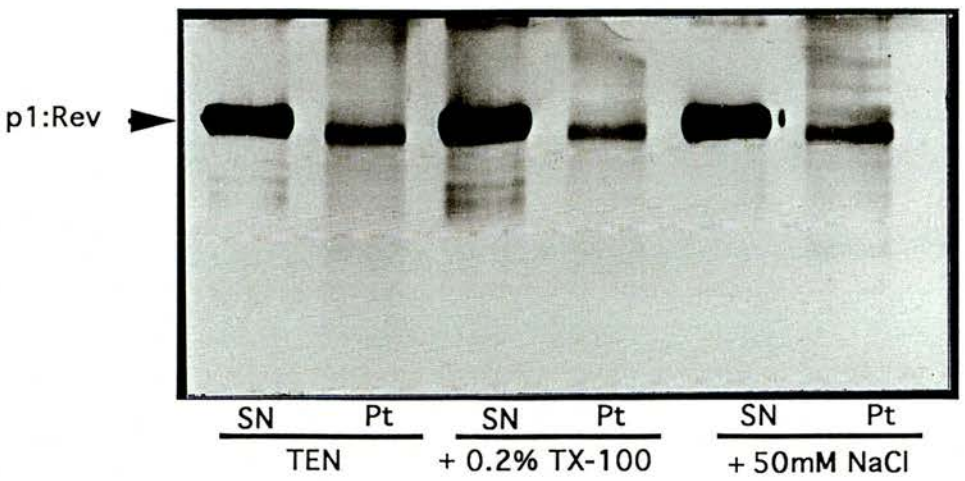
A SDS-PAGE and western blot analysis of the distribution of p1:Rev between pellet and supernatant fractions after post-lysis centrifugation. TRev.U yeast cells were incubated in Sc-glc-gal/trp for 24 hours and harvested. Lysates were prepared in TEN/pi (protease inhibitors) or TEN/pi supplemented with Triton X-100 (TX-100) or additional NaCl as indicated. An identical sample (0.25%) of each fraction was analysed. Samples were analysed by electrophoresis through a 10% polyacrylamide gel and blots probed with anti-p1 antiserum (1/1000 dilution).

B Recombinant Ty-Rev VLPs were prepared from supernatants derived from (A) above by centrifugation over 60% sucrose cushions. Centrifugation fractions (2ml) were collected and analysed by electrophoresis through 10% polyacrylamide gels. Gels were western blotted and probed with anti-p1 antiserum (1/1000 dilution). Numbers refer to fraction position after collection: 20 corresponds to the sucrose cushion. A sample of purified p1:p25^{gag} is included as an internal marker.

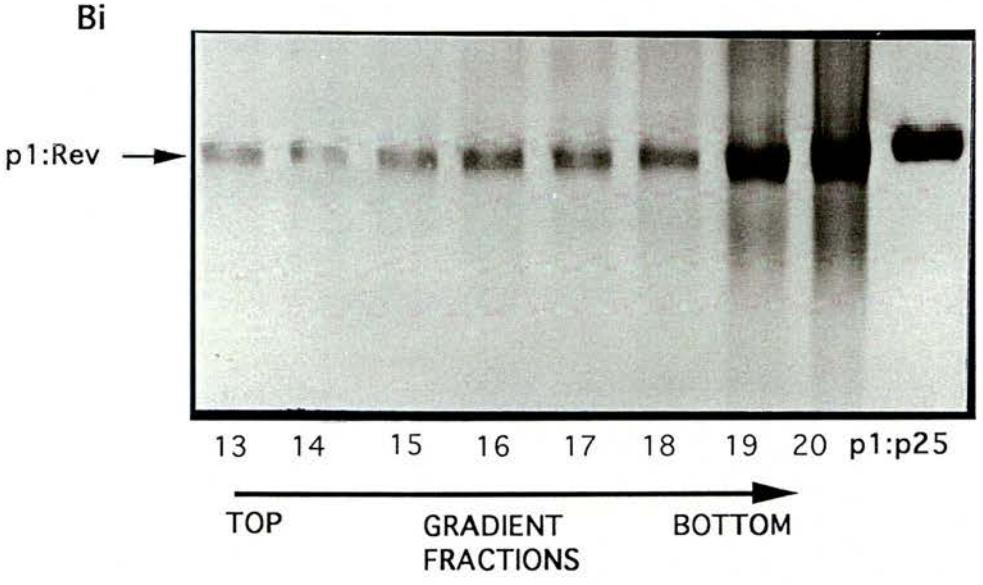
i Cells lysed in TEN/pi

ii Cells lysed in TEN/pi + 0.1% Triton X-100

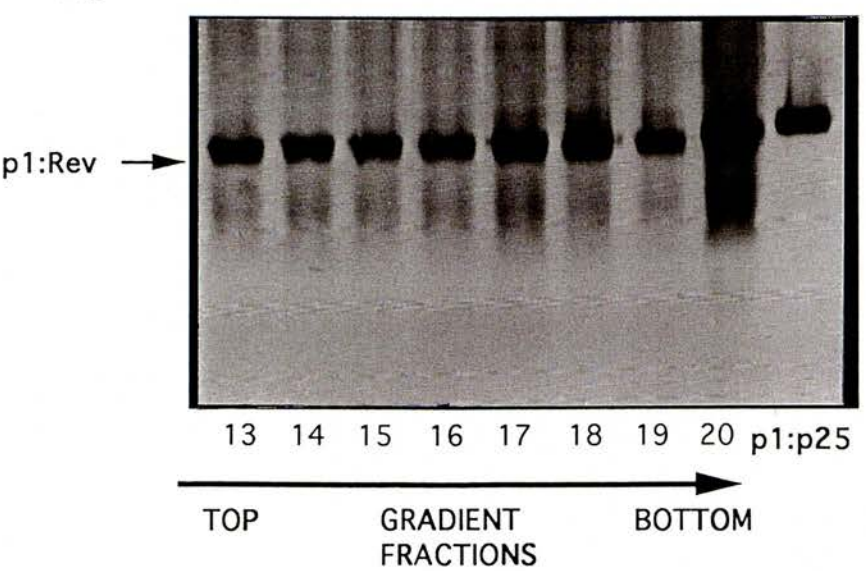
A


p1:Rev
SN
Pt
SN
Pt
SN
Pt
TEN
+ 0.2% TX-100
+ 50mM NaCl

Bi


p1:Rev
13
14
15
16
17
18
19
20
p1:p25
TOP
GRADIENT FRACTIONS
BOTTOM

Bii


p1:Rev
13
14
15
16
17
18
19
20
p1:p25
TOP
GRADIENT FRACTIONS
BOTTOM

cushion centrifugation was the second area of significant loss of protein. Western blot analysis of the distribution of p1:Rev after this 13000g centrifugation step demonstrates that most of the p1:Rev accumulates in the supernatant rather than in the pellet as expected (Figure 3.10a). Addition of the detergent Triton X-100 to 0.2% (v/v) of the cell extracts prior to centrifugation resulted in a reduction in the amount of residual p1:Rev within the pellet. This was not seen on the addition of 50mM NaCl. The value of this observation was reduced when it was observed that the presence of Triton X-100 within the sample pelleted onto the 60% sucrose cushion led to considerably less concentration of p1:Rev at the cushion interface than was otherwise the case, leading to a further loss of protein at this stage (figure 3.10b). Detergent was therefore not incorporated into the general purification protocol.

Sucrose cushion and sucrose gradient centrifugation also led to loss of p1:Rev. Selection of particular fractions enriched for VLPs meant that those particles which were distributed in less concentrated fractions were lost. This was an inevitable consequence of the purification procedure.

3.9.3 Alternative Purification Methods

3.9.3.1 Size Exclusion Chromatography

Chromatography was considered as a further purification step after gradient centrifugation of VLPs. The use of a Sephacryl S-1000 (Sigma) size exclusion column has been reported to enhance the purity of recombinant VLPs (Gilmour *et al.*, 1989). Preliminary investigations into the feasibility of such an approach using p1:Rev found that the volume of matrix required for purification was such that the relatively small number of VLPs would be massively diluted, and that adjustments to optimise chromatographic separation would be too time consuming to be justified.

Chromatography through sephadex G-25 was initially used to remove sucrose from selected cushion and gradient fractions as an alternative to dialysis, which was both time consuming and resulted in formation of a precipitate of yeast proteins which had to be removed by filtration. Pooled fractions from the sucrose gradient (13-16, volume 8ml) were applied to the column, and the flow through material collected in 2ml aliquots and analysed for sucrose content by refractometry, and for protein content by a dye-binding assay (Biorad). Figure 3.11a plots the concentration of sucrose and protein in each fraction. The distribution

3.11: Sephadex Chromatography

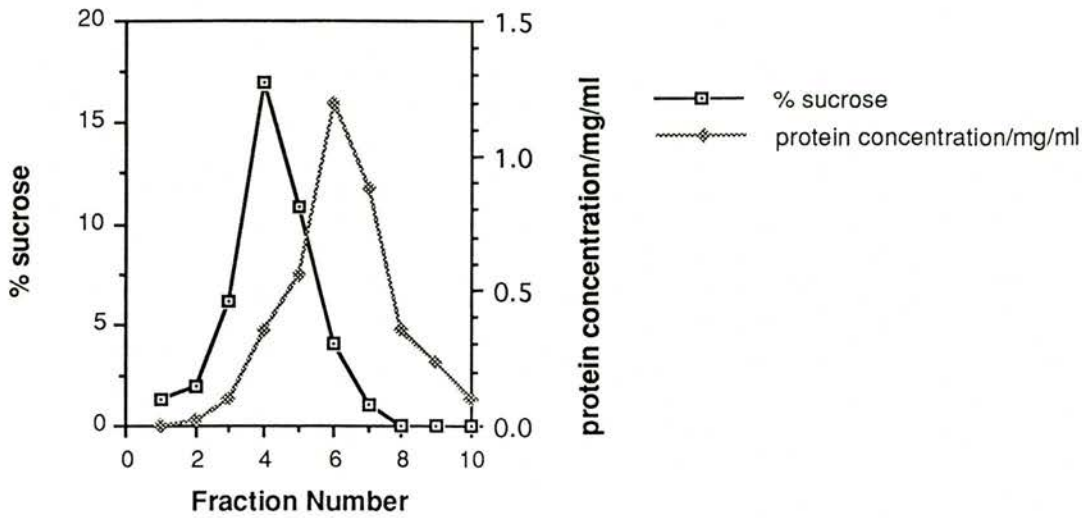
Fractions obtained from first round density centrifugation with peak p1:Rev concentrations were pooled and sucrose removed by column chromatography through Sephadex G-25. Run through fractions were collected in 2ml aliqouts and analysed for sucrose and protein content.

A Total sucrose and protein content of G-25 fractions. Sucrose content is plotted as a percentage for each sample: values obtained by refractometry. Pre-column material had a 22% sucrose content. Total protein content was calculated by Biorad protein concentration assay and is plotted in mg/ml.

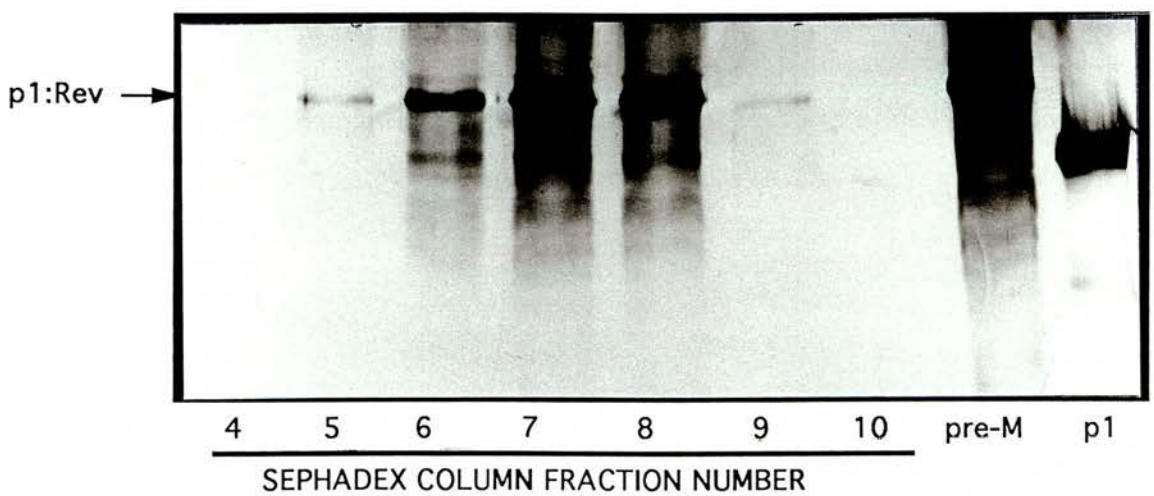
B Western blot analysis of the distribution of p1:Rev between Sephadex column fractions. Samples from each fraction (10µl) were resolved by electrophoresis through a 10% polyacrylamide gel and western blotted. Blots were probed with anti-p1 antiserum (1/1000 dilution). A sample of purified p1 was included as an internal marker. pre-M: Pre-column material.

C Coomassie stained PAGE profile of pre- and post-column fractions. pre-M: pre-column material. F7: fraction 7 from column run-through. A sample of p1:p25^{gag} was included as an internal marker.

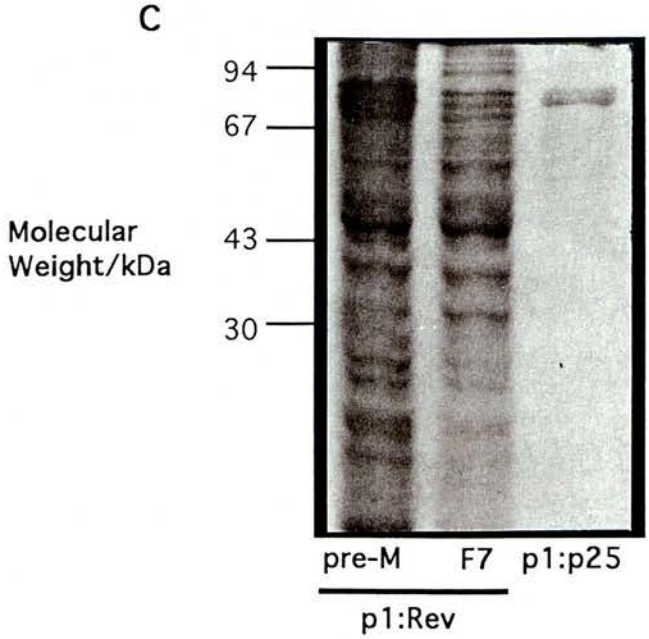
A


20
15
10
5
0
% sucrose
1.5
1.0
0.5
0.0
protein concentration/mg/ml
0
2
4
6
8
10
Fraction Number
% sucrose
protein concentration/mg/ml

B


p1:Rev
4
5
6
7
8
9
10
pre-M
p1
SEPHADEX COLUMN FRACTION NUMBER

C


94
67
43
30
Molecular
Weight/kDa
pre-M
F7
p1:p25
p1:Rev

of p1:Rev within the column fractions is illustrated by western blotting (Figure 3.11b). It was found that this procedure resulted in both a degree of concentration of the VLPs, and of purification. The peak fractions for VLP content (6-8, volume 6ml) are essentially free of sucrose (pooled sucrose content 1.5%). Furthermore, approximately 50% of total protein has been eliminated from these fractions, implying an increase in VLP purity. Examination of these fractions by Coomassie staining (figure 3.11c) however, demonstrates that this effect is not as marked as these figures would suggest. Fractions 6-8 were pooled and used for further study.

Partial proteolysis of the fusion protein to release the carrier p1 molecule may have occurred during chromatography, as evidenced by the appearance of blot-positive bands in the p1:Rev fractions migrating with the characteristics of p1. Possible contamination of the Sephadex with proteases may have caused this, and highlights the importance of maintaining conditions appropriate to protein stability during the purification process.

3.9.3.2 Immunoaffinity Chromatography

The methods of purification of p1:Rev outlined above are all based on the physical properties of the VLP. As has been demonstrated, the success of these methods is dependent on the yield of protein at the commencement of purification. To achieve high purity from a low yield preparation, a more specific selection process is required. The rabbit antiserum generated against p1 (section 3.8) was considered a suitable probe for the isolation of Ty:Rev VLPs. Coupling of antibody to an agarose based matrix allows for the isolation of antigen by antibody-affinity chromatography. This method has been shown to result in purification of protein essentially to homogeneity (Maze and Gray, 1980).

The γ-globulin fraction of R194 anti-p1 (α-p1) antiserum was purified by caprylic acid precipitation (2.24.2). After dialysis into a suitable buffer, the serum was coupled to Affi-gel 10 (Biorad). This form of crosslinked agarose contains an ester group which reacts specifically with ligands containing primary amino groups to form a covalent bond. Immunoglobulin G (IgG) molecules are thus immobilised by attachment to the affigel matrix. By monitoring the protein concentration of the serum before and after binding, the coupling efficiency was found to be 61%. This figure is relatively low compared to values quoted by the manufacturer (for example: 90% efficiency for human globulin). The affigel 10-αp1 was poured into a support

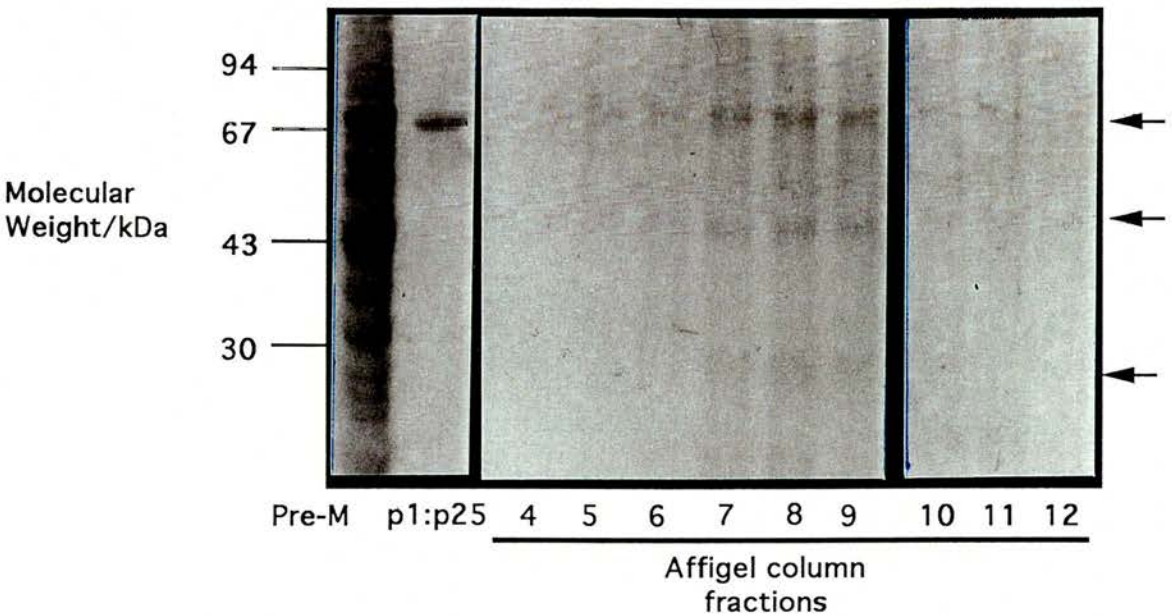
Figure 3.12: Purification of p1:Rev by Immunoaffinity Chromatography

An anti-p1 Affi-gel column was prepared as described (2.12.7.2.1). Fraction 14 (2ml) from the first sucrose gradient centrifugation step was applied and purification carried out as described (2.12.7.2.2). Eluted fractions (1ml) were collected and analysed by SDS-PAGE through 10% polyacrylamide gels and western blotting.

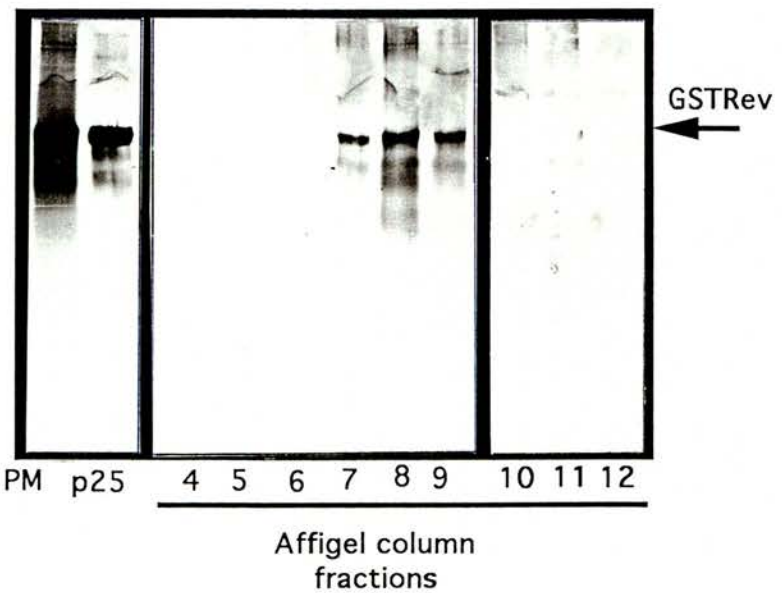
A Coomassie stained profile of SDS-PAGE analysis of chromatography fractions. Numbers refer to eluted fractions. Pre-M: pre-column material. A sample of p1:p25^{gag} was included as an internal marker. Arrows indicate protein bands discussed in the main text.

B Replica of the above gel was western blotted and probed with an anti-p1 antiserum (1/1000 dilution).

A


Molecular Weight/kDa
94
67
43
30
Pre-M
p1:p25
4 5 6 7 8 9 10 11 12
Affigel column fractions

B


GSTRev
PM
p25
4 5 6 7 8 9 10 11 12
Affigel column fractions

column, and p1:Rev containing material (primary sucrose gradient F14) adsorbed. Eluted fractions were tested for p1:Rev content by SDS-PAGE analysis. Fractions 5-11 contain protein as visualised by staining with Coomassie blue (Figure 3.12a). Three protein species are apparent, with molecular weights in the range 75-78, 50-55 and 25-30kDa. The largest of these migrates close to p1:p25^{gag}, and is a candidate for p1:Rev. This band is detected by immunoblotting with anti-p1 antiserum (Figure 3.12b) in fractions 7-9 only. This suggests that the band at 75-78kDa contains multiple species, although the one present in F7-9 may be predominant. Assuming this to be the case, the purity of p1:Rev as estimated from the Coomassie stained profile is ~40%. Although an obvious improvement on previous preparations, this result was disappointing. The nature of the contaminants is unknown. The lack of reactivity with anti-p1 antiserum rules out the possibility that degradation of the full length fusion protein is the source of these impurities: furthermore, as the probing antiserum is the same as that used in purification the contaminants cannot have been specifically recognised by the affigel 10-αp1. Non-specific antibody binding activity might account for the presence of contaminants, alternatively incomplete blocking of the affigel 10-p1 column might have led to binding of yeast extract proteins, although these would not be expected to then undergo elution. Alternatively, protein which is bound to p1:Rev may be retained on the column, and co-elute with p1:Rev. The presence of 140mM NaCl within wash solution might not be sufficient to dislodge specifically bound ligands. The efficiency of immunopurification of p1:Rev is impossible to gauge, given the lack of a reliable figure for the purity of p1:Rev in the pre-column material. The estimated total yield of p1:Rev eluted from the column was 20-25μg, leading to a final yield of ~5-6μg/L yeast culture. Although improvement in purity and yield of product might have been achieved by altering the conditions used, this figure was considered too low to justify further experimentation with this system. Use of the second gradient fraction 14 material as antigen might have increased purity of product, although the lower concentration of p1:Rev within this material might have reduced the yield of column product even further.

3.10 Expression of p1:Rev in an Alternative Host Strain

MC5 is a homozygous diploid strain (genotype: prb1, prc1, pep4, trp1, leu2, ura3) of

S. cerevisiae. (M. Cunningham, BBL, personal communication). Cells of this strain have a larger volume than haploid cells. It was hoped that this fact would allow these cells to withstand the toxic effect of expression of p1:Rev to a greater extent than the haploid BJ2168 cells, in turn leading to improved yield of protein.

MC5 cells were co-transformed by lithium acetate treatment with 5µg pRev.O and 5µg pUG41S. Double transformants (MRev.U) were visible after 4 days, although only 2-3 colonies per plate were obtained. Growth of MC5 transformants thus compared favourably to that of BJ2168 transformants (section 3.3). Selected transformants were used to seed small scale (100ml) cultures. Galactose induced cultures were monitored by light microscopy. There was some indication that MC5 transformants maintained higher viability during p1:Rev expression: however the proportion of dead cells was still high. Cell lysates were prepared and protein production analysed by SDS-PAGE. Immunoblotting with anti-p1 antiserum revealed the successful production of p1:Rev by the MC5 transformants. A similar level of protein is detected in each extract. No band due to p1:Rev is detectable when resolved extracts are stained with Coomassie blue (Figure 3.13a), however. By contrast, p1:p25^{gag} is clearly visible in a crude lysate. A time course study of the growth characteristics of MRev.U transformants, compared to TRev.U cells, was carried out as described in 3.6. The result is illustrated in figure 3.13b. It is clear that there is no significant difference between these yeast strains with respect to viability during p1:Rev expression.

Thus, although there may be a limited increase in p1:Rev tolerance associated with the diploid MC5 strain, this did not lead to an appreciable increase in protein yield. No further investigation into the effect of p1:Rev expression on MC5 growth was carried out.

3.11 Protease Cleavage of p1:Rev Fusion Protein

Although little data is available concerning the possible requirement for cleavage of fusion protein to generate biologically active recombinant proteins, cleavage of hybrid VLPs has been shown to be crucial for the activity of recombinant ovine interleukin-α and β (C. Fiskerstrand, PhD thesis, 1994). Sucrose-gradient purified p1:Rev protein was tested for reactivity with Factor Xa in the hope of generating a Rev polypeptide which might then be purified away from contaminating yeast material. The results of cleavage were analysed by probing western blots with anti-p1 antiserum, and with the anti-p1:Rev antiserum R194 (3.8)

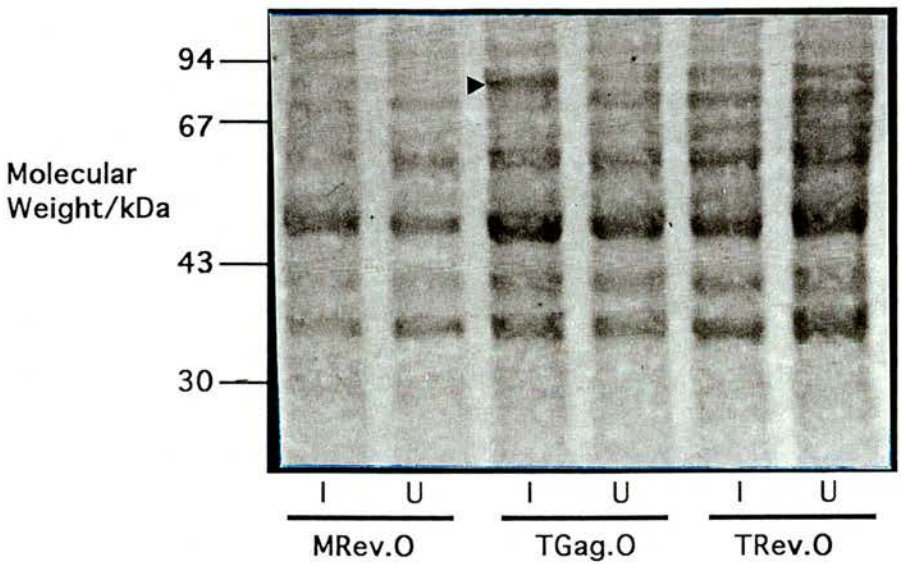
Figure 3.13: Expression of p1:Rev in an Alternative Yeast Host Strain

The vectors pRev.O and pUG41S were transformed into *Saccharomyces cerevisiae* strain MC5. Transformants (MRev.U) were used to seed small scale (100ml) cultures. Cultures of BJ2168 cells co-transformed with pUG41S and pRev.O and pGag.O (expressing p1:p25^{gag}) (TRev.U and TGag.U respectively) were also grown. Cultures were split, and half used to induce fusion protein expression by incubation in Sc-glc/gal/trp. Cells were harvested after 24 hours, and extracts prepared by lysis.

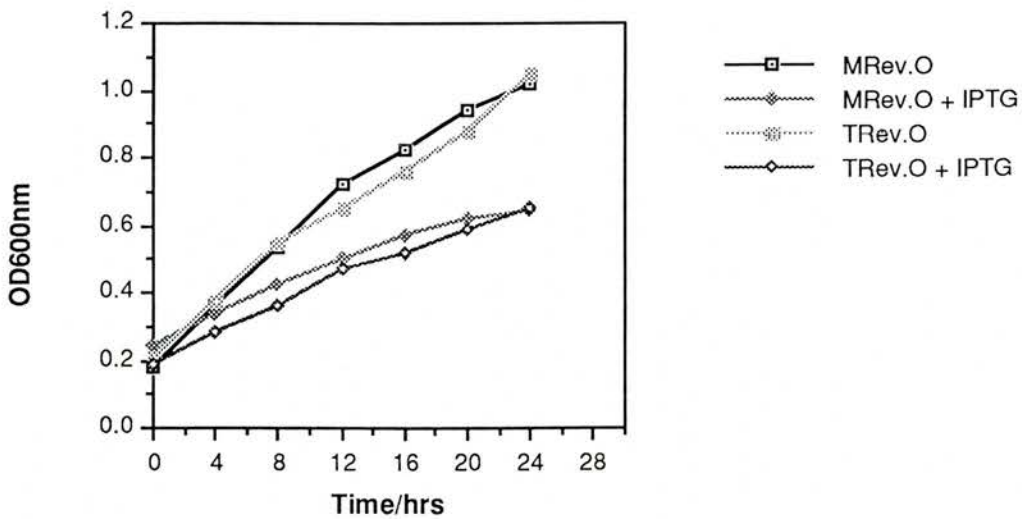
A Cell extracts (5μl) were analysed for protein content by SDS-PAGE through a 10% polyacrylamide gel. Proteins were visualised by staining in Coomassie blue. U: uninduced cultures, I: induced cultures. The band corresponding to p1:p25^{gag} is arrowed.

B Time course study of culture growth. The optical density at 600nm (OD_{600nm}) of the TRev.U and MRev.U cell cultures described above, was recorded at intervals of 4 hours.

A


94
67
43
30
Molecular Weight/kDa
I U I U I U
MRev.O
TGag.O
TRev.O

B


1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD600nm
0 4 8 12 16 20 24 28
Time/hrs
MRev.O
MRev.O + IPTG
TRev.O
TRev.O + IPTG

in an attempt to identify cleaved product. The extent of cleavage was monitored by the loss of the p1:Rev band at 75kDa, and its replacement with a p1 band at 55kDa (Figure 3.14a). It can be seen that the reaction proceeds efficiently, with cleavage to completion after two hours incubation at an enzyme concentration of 1% of that of the substrate. The reactivity of the anti-p1:Rev antiserum R206 with contaminating yeast proteins makes interpretation of this blot (3.14b) difficult. The pattern of smaller, blot-positive proteins alters between the cleaved and uncleaved samples, perhaps reflecting protease activity on the contaminants. No Rev-specific band can be allocated. Silver staining a replica of this gel gave a similarly confusing result, with no evidence of a potential Rev polypeptide. The failure to detect a blot-positive Rev band may not be surprising, given the incomplete characterisation of R190 antiserum with respect to anti-Rev activity (3.8), however the lack of a silver stained product suggests that the cleaved polypeptide may be unstable, leading to complete proteolysis.

3.12 Discussion

This chapter has reported the generation of plasmids to direct the expression of recombinant MVV EV1 Rev protein as a fusion to the p1 component of yeast Ty-VLPs. Although expression was achieved, the yield and purity of product were found to be low in comparison both to native p1 and to several other hybrid VLPs. The purification process is dependent on the particulate nature of the hybrid VLPs, and thus involves non-specific enrichment of VLP containing material, rather than protein-specific procedures. This has the advantages of rapidity and versatility, but is relatively inefficient and is dependent on a high initial yield of product. The low yield of p1:Rev achieved meant that low purity was inevitable, such that even two rounds of sucrose gradient enrichment resulted in only 10% purity at best.

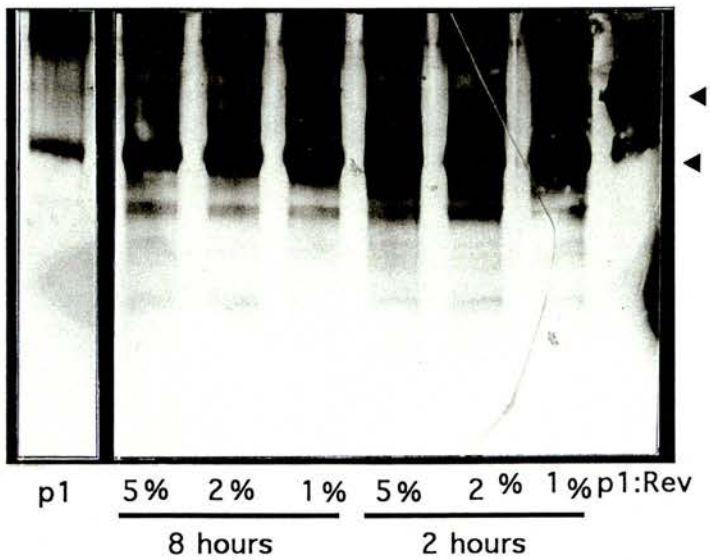
3.12.1 Toxicity of p1:Rev

The growth of cell cultures was quickly curtailed on induction of p1:Rev expression. Microscopic examination of cells showed that this was due to cell death rather than cytostasis. No such effect was observed in cells expressing native p1 protein, implicating the Rev-specific region of the fusion protein in p1:Rev toxicity. There are several indications that the toxic mechanism is specific, resulting in cytocidal effect at low concentrations. These include

3.14: Factor Xa Digestion of the p1:Rev Fusion Protein

Aliquots (25ml) of p1:Rev containing samples from second sucrose gradient fraction 14 were incubated with the protease Factor Xa for two or eight hours as indicated, at a range of concentration ratios (approximately 1-5% w/w enzyme to substrate). Reaction conditions as described in 2.14. After incubation, samples were boiled in SDS-PAGE loading buffer to stop reactions, and resolved by electrophoresis through 10-20% gradient polyacrylamide gels. The gels were western blotted and probed with rabbit anti-p1Rev antiserum.
The relative positions of p1 and p1:Rev are marked by arrows.

A



the rapid reduction in growth on induction of p1:Rev expression and the low yield of protein obtained. Cells doubly transformed with a pOGS40-based vector and the helper plasmid pUG41S have been shown to yield six-fold more fusion protein than single transformants (Reyburn *et al.*, 1992). The lack of a significant difference in terms of growth and yield between such transformants observed here suggests that toxic effect is manifest at an early stage of expression and at low fusion protein concentrations.

The actual mechanism of Rev toxicity is open to speculation. Although toxicity at the mRNA level cannot be dismissed, it seems probable that toxic effect is mediated by the protein. Direct effects of Rev itself are the probable cause of toxicity, although the role of Rev-induced modifications to the structure of p1 within the context of VLPs cannot be ruled out. The failure of attempts to express the HIV-1 Rev protein in the Ty-VLP system due to toxic effect (S. Adams, BBL, personal communication) suggests that regions of homology between HIV-1 and EV1 Rev proteins may be responsible for toxicity. Such regions are the two conserved functional domains, containing putative RNA and protein binding motifs (3.2). Thus, toxicity may be the result of non-specific, large scale binding of Rev to yeast RNA, resulting in reduced expression of cellular genes and/or disruption of ribosomal structure. Alternatively, or in concert, binding of the leucine-rich motif to cellular proteins may be important. Specific binding of HIV-1 Rev leucine-rich domain to the human protein eukaryotic translation initiation factor 5A (eIF-5A) has recently been demonstrated (Ruhl *et al.*, 1993). This factor is highly conserved amongst eukaryotes, such that human eIF-5A can functionally substitute for the homologous yeast gene *HYP2* (Koettnitz *et al.*, 1995). Cellular depletion of this factor has been shown to result in immediate inhibition of the growth of cultures of *S. cerevisiae* (Kang and Hershey, 1994), although these authors did not record whether this was a consequence of cell death. It is therefore possible the Rev-mediated sequestration of pHYP2/eIF-5A is at least partially responsible for cytotoxicity. Other cellular factors could also be sequestered in this manner.

The conformation of Rev may be significant. One possibility is that toxicity is apparent before assembly of p1:Rev monomers into VLPs; this would explain the low density of VLPs evident in expressing cells by electron microscopy. However, western blot data demonstrates that most of the fusion protein extracted from cells behaves as particles during density centrifugation. It is also possible that the reverse is true, ie that particle formation is a prerequisite for toxicity. Expression of native HIV-1 Rev protein in *S. cerevisiae* cells has not

been associated with toxicity (Stutz and Rosbash, 1994), whereas Ty:HIV-1 Rev demonstrates toxicity (S. Adams, BBL, personal communication). Regardless of their nature, the formation of VLPs consisting of ~300 p1:Rev monomers would result in extensive cross-linking of possible Rev ligands within the cell. If the ligands were components of cellular structures such as the ribosomes or nuclear membrane, then cross-linking might cause the observed disruption in yeast morphology. Vacuolation of host cells could occur as a consequence of disruption. Recently, a putative component of the yeast nuclear pore complex, Rip-1p, has been shown to specifically interact with the HIV-1 Rev activation domain (Stutz *et al.*, 1995). Although these authors demonstrate that depletion of this factor does not significantly reduce cell viability, Rev cross-linking of this molecule might cause damage to the cell ultrastructure. Evidence for the interaction of p1:Rev with cellular macromolecules is available in the behaviour of p1:Rev during density centrifugation, and possibly, in the co-purification of contaminating molecules during affinity chromatography. Addition of the detergent Triton X-100 altered the pattern of p1:Rev distribution, reducing the concentration in the lower half of the gradient. As one effect of detergents is to reduce binding between proteins, this would argue that hybrid VLPs were found in the form of complexes under normal conditions. Furthermore, the gradient fractions containing the highest purity of p1:Rev after two rounds of centrifugation include more contaminants than are present in a similarly obtained preparation of native p1. p1:Rev induced aggregates are the likely source of these co-sedimenting proteins.

HIV-1 Rev is functional in yeast cells (Stutz and Rosbash, 1994). This implies that the known characteristics of HIV-1 Rev, such as nuclear import and multimerisation, can occur in yeast cells. Although there was no evidence by electron microscopy for Ty:Rev VLP entry into yeast nuclei, disruption of cellular morphology meant that definite conclusions as to subcellular particle distribution could not be drawn. Specific entry of the large, polymeric VLP into the nuclear pore may not occur; however, entry of p1:Rev monomers might occur. This would allow the expression of possible nuclear toxicity. Blocking of nuclear pores by recombinant VLPs may also be a significant factor in the toxic effect.

Further study would be required to confirm the role of Rev in toxicity. Indeed, the tractability of the yeast system could allow more detailed investigation into the nature of Rev binding. Determination of the involvement of conserved domains of Rev in toxicity by engineering mutations into the expression plasmid could lead onto identification of cellular

proteins bound by Rev. Subcellular localisation of Ty:Rev VLPs within yeast cells by immunofluorescence would determine whether cellular aggregates are associated with these particles.

Diploid MC5 cells were used in an attempt to circumvent problems of toxicity. That these larger cells appeared to survive slightly better than the haploid hosts lends credence to the toxic hypothesis outlined above. However, toxicity was sufficiently high to ensure that protein yield was not significantly increased by expression in this strain.

One problem associated with the regulatable pOGS-based vector was the level of pre-induction expression directed by the PAL promoter. This caused reduced growth of pre-induced cells, leading to problems in increasing culture biomass before fusion protein expression. Neither the background level nor the galactose induced percentage increase in PAL transcription have been documented. However, transcription from the PGK promoter is reduced, but not abolished, by removal of the UAS-PGK (Ogden *et al.*, 1986). A recently developed promoter, pJC78, contains the upstream repression sequence from the GAL 1/10 promoter, and should therefore be tightly repressed in the presence of glucose (M. Cunningham, BBL, personal communication). Use of this promoter might allow improved Rev expression by increasing the number of healthy cells prior to induction; however, any such improvement is likely to be slight given the extremely toxic nature of p1:Rev expression.

It was noted that, even after 24 hours incubation in galactose, a small proportion of yeast cells retained normal morphology. It is possible that these cells were incompletely induced and had not expressed sufficient fusion protein to experience toxic effect. An alternative explanation is that the severe nature of toxicity created a strong selective pressure for reduction in copy number, total loss or mutation of the expression plasmid, such that growth in the absence or reduced concentration of the selected nutrients was favoured over growth in the presence of p1:Rev. The consequences in terms of yield of fusion protein of reduced copy number are likely to be masked by the toxicity of expression.

3.12.2 Particle Formation

Early investigation into the nature of hybrid Ty-VLPs supported the hypothesis that

the foreign polypeptide was expressed at the surface of the particle (Adams *et al.*, 1988). This, in turn, led to the assumption that hybrid Ty-VLPs were ideal immunogens, presenting multiple copies of the foreign protein in an accessible, particulate form. The Ty:HIV-1 $p24^{gag}$ VLP was shown to elicit a strong anti-p24 immune response (Gilmour *et al.*, 1989) and, by immunogold analysis, to present p24 on the surface of particles (Adams *et al.*, 1994). Particles composed solely of p1 demonstrate diversity in size but retain symmetry, suggesting that assembly occurs by an ordered, but flexible, procedure (Burns *et al.*, 1992). However, many hybrid particles possess the irregular morphology demonstrated by Ty-Rev VLPs, for example Ty:MVVgp46 (C. Cousens, PhD thesis, 1994) and Ty:MVV gp135 (residues 1-311)(Carey *et al.*, 1993). In addition, immunisation with these and other hybrid particles has been found to mimic the response to Ty:Rev, by the development of strong anti-p1 responses, but little or no response to the foreign antigen (Reyburn *et al.*, 1992; Fiskerstrand *et al.*, 1993). A possible explanation for these findings is forthcoming from the recent study of the detailed structure of native VLPs by Brookman and co-workers (1995). Using monoclonal antibodies directed against known p1 epitopes, these authors determined that the carboxy-terminal region of p1 is buried within the VLP, and that the amino-terminal region projects from the surface. As the Ty-VLP expression system is based on carboxy-terminal fusions, then the proposed model of hybrid particles is unlikely to be accurate. Although the mechanism of particle formation is unknown, the presence of significant additions to the carboxy-terminus is likely to cause extensive disruption, explaining the heterogeneity of hybrid VLPs. The location of the foreign protein within hybrid VLPs may be an important factor in determining both immunogenicity and biological activity. An internal location for the foreign polypeptide is the likely reason for poor immunogenicity. It should be noted that amongst those hybrid particles which do function well as immunogens, several, including Ty:HIV-1$p24^{gag}$ (Gilmour *et al.*, 1989) and Ty:SIV$p27^{gag}$ (Lehner *et al.*, 1994) contain added proteins with intrinsic assembly determinants, which might compete with those of p1 to alter the structure of the particle. Thus, the arrangement of proteins within the particle may be the result of higher order structural interactions between the carrier and added protein, and may be different for each hybrid. If multimerisation is a property shared by MVV and HIV-1 Rev proteins, then intramolecular interactions between the Rev moieties might interfere with particle assembly. The heterogeneity in particle size observed suggests that there may be variation in particle

structure between hybrid Ty-Rev VLPs, which might support this hypothesis.

There is conflicting evidence concerning the location of Rev within the Ty:Rev particles. Efficient cleavage and the specific toxic effect of expression suggest that Rev is largely or completely superficial in character (although the detergent utilised in proteolysis might disrupt native structure). However, the lack of an apparent anti-Rev immune response argues for masking of Rev by p1. It may be that the structure is complex, with certain residues of Rev exposed and others hidden within the particle. A similar analysis to that of Brookman *et al* (1995) would be required to determine the actual structure of Ty:Rev VLPs.

3.12.3 Improved Expression of Rev in Yeast

The precise structure of the Ty:Rev VLP is of importance to the success of affinity-gel purification. The antiserum used for purification must recognise the hybrid particles in their native form. Use of the anti-p1:Rev antiserum (R190) for purification was counter-intuitive, since this serum was developed against the same sucrose gradient material to be purified. The anti-p1 antiserum (R194) was therefore used. Immunisation with the p1:Rev material induced a strong anti-p1 response, implying that p1 has at least a degree of surface localisation. Thus, purification of Ty:Rev VLPs with anti-p1 antiserum should be feasible. However, a relatively poor result was achieved using this approach. It is possible that the p1 epitopes recognised by R194 are mostly hidden within the Ty:Rev particle, reducing the effectiveness of purification. Purification would be improved by use of an antiserum directed specifically against Ty-Rev VLP surface epitopes.

Other aspects of affinity gel purification were subject to improvement. The coupling efficiency of antibody was relatively low. The binding of affigel is dependent on the buffering of the ligand. Affigel 10 has a slight negative charge, and binds to neutral and basic proteins. If components of the polyclonal antiserum possessed isoelectric points below the pH of the buffer used (7.2), then they would assume a negative charge and bind poorly to the affigel, thus resulting in a low overall efficiency of coupling. Changing the buffer system might boost coupling. Altering the elution conditions might also be beneficial; Ikeda and Steiner (1976) achieved purification of platelet-derived tubulin from antibody linked to affigel 10, to 96% by eluting in 0.05% Triton X-100/0.1M phosphate buffer, pH 7.0. Use of a monoclonal antibody

would allow more precise optimisation of conditions.

Other strategies to improve expression would involve combating the problem of fusion protein toxicity. Replacing the PAL promoter with one more tightly repressed in the absence of galactose has already been discussed. Use of an alternative yeast strain has been attempted here with little success. Other, more genetically diverse, strains might prove useful. Another approach would be to substitute the Ty-VLP system for one based on the secretion of heterologous proteins from host cells, in the hope that toxicity could thus be by-passed. Many of these systems are based on fusion to the leader sequence of the yeast mating hormone α-factor (reviewed by Brake, 1990).

3.13 Summary

A *rev* gene derived from EV1, a British isolate of the ovine Maedi Visna Virus, was cloned and sequenced. Regions of homology to the partially characterised HIV-1 Rev protein were identified. Vectors to direct the expression of the Rev protein as a carboxy-terminal fusion to a modified form of the p1 protein of the *S. cerevisiae* retrotransposon Ty, were constructed. Transformed cells produced p1:Rev in the form of hybrid particles, detected by western blotting and electron microscopy. Both the yield and purity of p1:Rev were found to be lower than expected. Examination of the growth profiles of expressing yeast demonstrated that p1:Rev production was associated with inhibition of culture growth. Dramatic deleterious effects of p1:Rev on yeast morphology were observed. Conventional purification of hybrid VLPs was largely unsuccessful, due to the requirement for high yield for high purity. Some improvement in culture condition was obtained by altering growth conditions. Product purity was enhanced by immunopurification with an anti-p1 antiserum; however overall yield was further reduced. Immunisation of a rabbit with p1:Rev preparation elicited a strong anti-p1 response, but no detectable response against Rev. It was decided that this system was not appropriate for the production of Rev protein for functional studies.

CHAPTER FOUR

EXPRESSION OF EV1 REV IN THE BACTERIAL pGEX SYSTEM

4.1 Introduction

Chapter 3 outlined the production of recombinant Rev protein in the yeast Ty-VLP system. It was shown that Rev-induced cytotoxicity resulted in low yields of Ty:Rev fusion protein. Fusion protein purity was also found to be low, due to the nature of the purification process. An alternative expression strategy was therefore sought. It was hoped that the use of a prokaryotic expression system would circumvent problems of toxicity present in yeast. The pGEX system was chosen as several requirements for the production of biologically active proteins are reported features of this system. These typically include high yield, good solubility and a rapid and efficient purification protocol based on affinity binding without the need for denaturing conditions, thus preserving the antigenicity and functionality of the recombinant protein.

Foreign polypeptides are expressed in the pGEX system as carboxy-terminal fusions to a modified form of Sj26, the glutathione-S-transferase (GST) enzyme of the parasitic helminth *Schistosoma japonicum* (Smith *et al.*, 1986). The original expression vector pGEX1 has been modified to incorporate additional features to aid cloning and expression: the derivative vector pGEX2T is illustrated by figure 4.1. The foreign gene is inserted at the 3' end of *gst*. Expression is under the control of the *tac* promoter (P_{tac}), a hybrid derived from the *trp* and *lac* promoters (Amann *et al.*, 1983; De Boer, *et al.*, 1983). P_{tac} is constitutively repressed by the product of the *lacI*^{q} gene, but directs high level transcription on induction with the de-repressor, IPTG. Constitutive repression and inducible expression is an important feature when dealing with a potentially toxic protein (Brosius, 1984). The presence of the *lacI*^{q} gene within the vector means that expression is independent of host genotype.

The polylinker region at the cloning site varies in reading frame between the plasmids. In the derivative vectors pGEX2T and pGEX3X the polylinker region also encodes several extra amino acids which form protease cleavage sites. These allow removal of the protein of interest from the carrier. The polylinker is followed by translational termination codons in all three reading frames.

After induction of expression for a suitable period, transformed cells are harvested and a cell lysate prepared by sonication. The fusion protein is isolated by specific binding of the GST carrier to glutathione immobilised by covalent attachment to agarose beads. After

Figure 4.1: The Expression Vector pGEX2T

The diagram shows the map of the vector pGEX2T. The cloning site of the vector is depicted below. The restriction enzyme recognition sequences, encoded amino acid sequence and the position of thrombin cleavage site are marked.

P_{tac}: the hybrid *tac* promoter. Directs inducible, high level transcription

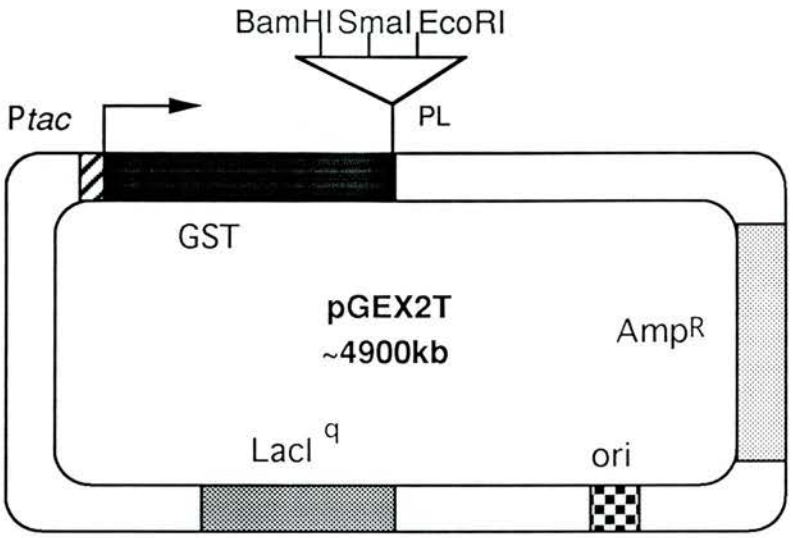
GST: modified form of the glutathione-S-transferase gene (Sj26) of *Schistosoma japonicum*

PL: polylinker region

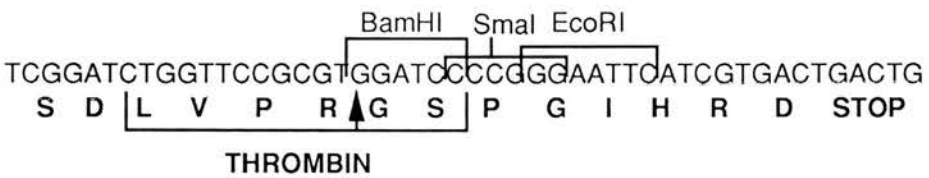
Amp^R: ampicillin resistance gene

ori: origin of replication

$LacI^q$: lac repressor gene. The product binds to, and suppresses, P_{tac}



Polylinker Region



washing, the fusion protein is collected by elution from the beads through competition with free, reduced glutathione.

Successful protein expression in the pGEX system has been widely reported. Fusion proteins have been used for immunological (Frosch *et al.*, 1991), vaccine (Fikrig *et al.*, 1990) and functional studies (Kaelin *et al.*, 1991). Indeed, in many cases the full fusion protein demonstrates correct activity, obviating the need for proteolysis to remove the carrier (Plutzky *et al.*, 1992). However, in some cases cleavage of the recombinant protein is required to release activity (Gearing *et al.*, 1989). Fusion proteins with total molecular weights from 32kDa to 84kDa have been expressed (Smith and Johnson, 1988). Typical yields of recombinant protein are within the range 1-5mg/L bacterial culture (Smith and Corcoran, 1990), though exceptionally yields as high as 20-80mg/L have been obtained (Frorath *et al.*, 1991).

The main problems encountered using this system, as with other bacterial expression systems, have involved fusion protein solubility and stability. High level expression of proteins in *E. coli* often leads to sequestration in insoluble inclusion bodies. This is not the case with native GST, which remains in a soluble state even when expressed at levels up to 15µg/ml bacterial culture. It was initially suggested that the majority of proteins expressed in pGEX would be soluble (35/47 expressed proteins, Smith and Johnson, 1988). More widespread recent use of this system has demonstrated that insolubility is actually commonplace (Frangioni and Neel, 1993). However, it has also been shown that detergent-mediated solubilisation of fusion protein inclusion bodies can work efficiently to produce pure, functional protein. Fusion protein instability can also be problematic. Factors governing stability are poorly understood, though it has been suggested that host bacterial genotype can play a role in determining stability (Smith and Corcoran, 1990).

4.2 Generation of a Polyclonal Anti-Rev Antiserum

In order to aid purification of the recombinant Rev protein, it was decided to raise an anti-Rev antiserum by utilising a synthetic peptide corresponding to a sequence motif within the EV1 *rev* gene. It was hoped that this would generate a more reliable reagent than the anti-p1:Rev serum, R190.

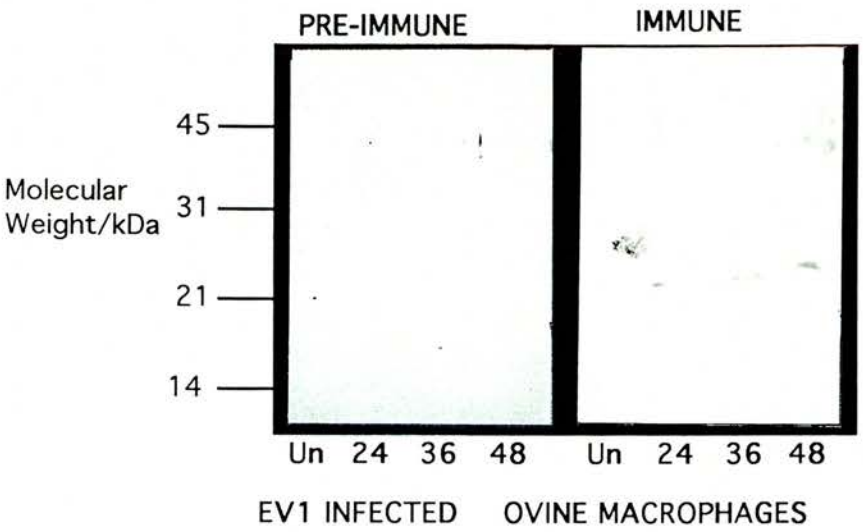
The central, highly basic region of the EV1 Rev molecule is the most antigenic, according to computer prediction. However, technical difficulties were encountered in the

4.2: Characterisation of an Anti-Rev Peptide Antiserum

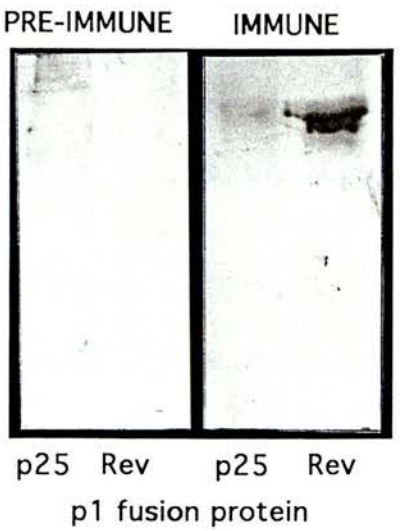
A Extracts of ovine macrophages prepared at several time points post-infection with MVV strain EV1, were obtained from Dr. D. Roy, Department of Veterinary Pathology, University of Edinburgh. These were resolved by SDS-PAGE and Western blotted. Blots were probed with rabbit (R206) serum obtained prior to or 24 days after immunisation with a peptide derived from the EV1 Rev sequence (1/100 dilution). Un: uninfected cell extract, numbers refer to hours post-infection.

B Western blot analysis of yeast samples containing p1:Rev and p1:$p25^{gag}$ (chapter 3). Blots were probed with pre-immune or immune sera from R206 as above (A).

A


PRE-IMMUNE
IMMUNE
45
31
21
14
Molecular Weight/kDa
Un 24 36 48
Un 24 36 48
EV1 INFECTED OVINE MACROPHAGES

B


PRE-IMMUNE
IMMUNE
p25 Rev
p25 Rev
p1 fusion protein

synthesis of this arginine-rich peptide. A sequence overlapping the carboxy-terminal region of EV1 *rev* with homology to the activation domain of the HIV/SIV Rev proteins was chosen as an alternative antigen. The sequence, amino acid residues 101-119, is as follows:

101 **ESNVAGLEKLTLEEKLEEKC** 119

The carboxy-terminal cysteine residue does not appear within the protein sequence, and was added to allow peptide conjugation to ovalbumin, to generate a more immunogenic reagent. Conjugation was mediated by the bifunctional cross-linking reagent, 3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide (SPDP) which forms disulphide bonds with cysteine residues. The peptide was synthesised by Mr. D Thomson, Department of Veterinary Pathology, University of Edinburgh. A polyclonal antiserum was raised as described in section 2.24.1. To test for anti-Rev reactivity, both pre-immune and immune sera were used to probe Western blots (figure 4.2). The immune serum is reactive with a protein of apparent molecular weight 22-23kDa present in EV1-infected ovine macrophages (4.2a): this reactivity does not extend to uninfected cells. This band is assumed to represent Rev, which may migrate more slowly than its calculated molecular weight of 19.5kDa suggests (see section 3.3). Moreover, the immune serum specifically recognises a band corresponding to p1:Rev in a TRev.U yeast cell extract (figure 4.2b). Thus the anti-Rev activity of this serum was confirmed.

4.3 Construction of Recombinant pGEX:Rev Expression Plasmids

The recombinant pOGS-derived plasmids used for expression in the yeast Ty system were a convenient source of *rev* gene insert. Both pOGS and pGEX contain a BamHI site within the polylinker region, enabling the *rev* insert to be transferred directly. Vector pGEX2T was chosen to maintain the correct reading frame. An additional benefit of using the pOGS-based insert was the presence of a protease Factor Xa site within the gene fragment. This led to the presence of two different enzyme sites within the GST/Rev adapter region.

The *rev* gene fragment (520bp) was excised from plasmid pRev.O (3.3) by digestion with BamHI, isolated by agarose gel electrophoresis and purified by hot phenol extraction. Ligation into the BamHI, CIP treated vector pGEX2T (a kind gift of Dr. Donald Smith) was carried out at approximate molar ratios of 1:2, 1:1, 2:1. Ligation products were transformed

Figure 4.3: Restriction Digest Analysis of pGEX2TRev Transformants

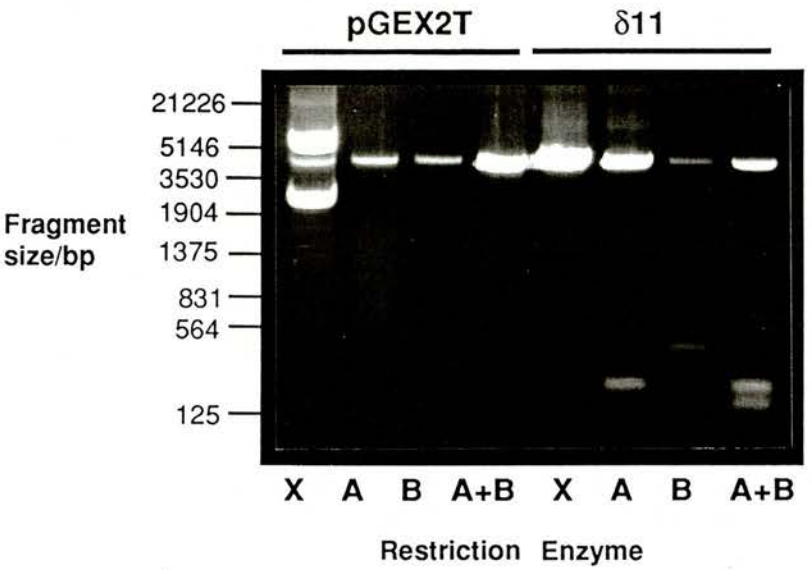
Plasmid DNA was prepared from several pGEX2T/rev insert transformants. Restriction digest analysis of one of these (δ11) is shown. Analysis of the original pGEX2T vector is shown for comparison.

Restriction enzymes used:

A: AvaI B: BamHI X: XhoI

Predicted restriction fragment sizes (bp)

Restriction Enzyme	pGEX2T	pGEX2TRev (correct orientation)	pGEX2TRev (inverted orientation)
AvaI	4900	302	218
BamHI	4900	520	520
XhoI	uncut	5420	5420
AvaI +BamHI	4900	302,218	218,302


pGEX2T
δ11
21226
5146
3530
1904
1375
831
564
125
Fragment size/bp
X A B A+B X A B A+B
Restriction Enzyme

into competent *E. coli* JM101 cells, and transformants selected on the basis of ampicillin resistance.

A small number (10) of transformants was obtained. The colonies were screened for the presence of an insert of the correct molecular size by BamHI restriction digest of plasmid minipreparations. Analysis of one of the colonies, δ11, is shown in Figure 4.3. Insert identity was confirmed by the susceptibility of the plasmid to digestion with XhoI, a recognition site for which is present in the *rev* gene but not in pGEX2T. Insert orientation was determined by the presence of AvaI sites within the insert (position 218) and immediately 3' to the BamHI insertion site in the vector. Double digest with BamHI and AvaI produced an additional smaller fragment (218bp) compared to digest with AvaI alone, when the insert was present in the correct orientation.

4.4 Validation of Transformants for Expression of Fusion Protein

Four transformants were selected on the basis of restriction digest analysis and were used to prepare small scale cultures (2ml). Bacteria transformed with pGEX2T alone were used as controls. Cultures were induced with IPTG for three hours, the cells harvested and fusion proteins purified by incubation of cellular lysates with glutathione-agarose beads. Purified proteins were resolved by SDS-PAGE and visualised by silver staining (Figure 4.4a). A band migrating with an apparent molecular weight of 45-48kDa was found to be present in the purified extracts from all four potential pGEX2TRev transformants. This size is broadly consistent with the predicted molecular weight of GSTRev (47kDa: GST 27.5 + Rev 19.5). Although evidence from figure 4.2 suggests that free Rev migrates with an apparent molecular weight of 22-23kDa, the presence of the GST carrier may alter mobility. A group of proteins with molecular weights in the range 28-34kDa are also present in the purified material. Cells transformed with pGEX2T produce native GST which was found to migrate with a molecular weight similar to its predicted value of 27.5kDa. It should be noted that there is a considerable reduction in yield of GSTRev compared to GST.

To confirm the identity of the 45-48kDa protein as GSTRev, a Western blot of the above gel was probed with the anti-Rev peptide antiserum. The result of this blot is illustrated by figure 4.4b. Only the 45-48kDa protein is recognised by the antiserum. Neither native GST nor the cluster of contaminating proteins are blot positive.The 45-48kDa protein is thus a

Figure 4.4: Analysis of Protein Expressed by pGEX Transformants

A Protein was purified on glutathione-agarose beads from lysates of cultures of each of the bacterial clones shown. Proteins bound to the beads were resolved on a 10% SDS-PAGE gel, and visualised by silver staining. The putative GSTRev protein is indicated by an arrow.
Clones: pGEX2TRev transformants: β2 β4 δ11 δ22
pGEX2T transformants: α6 α9

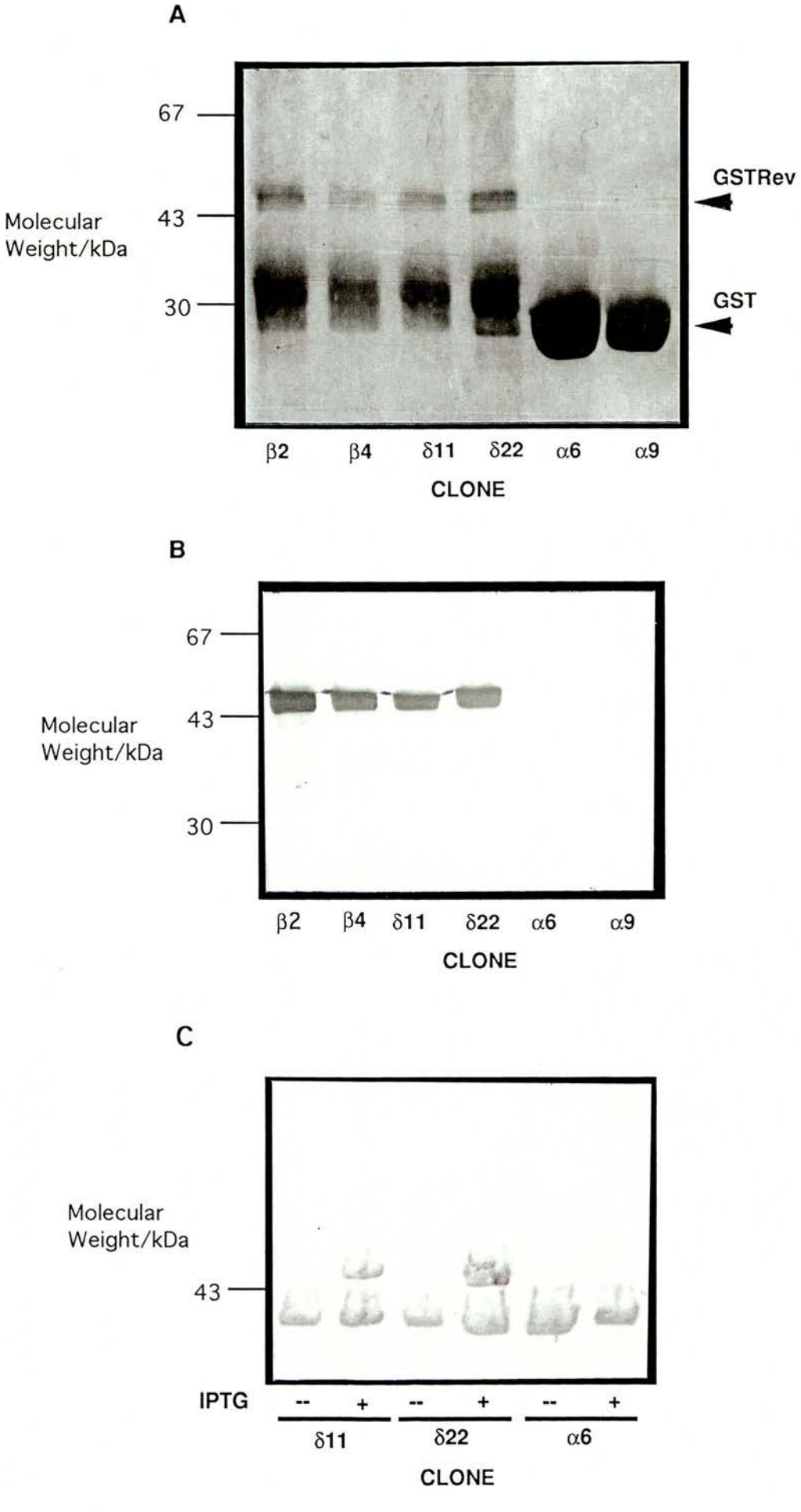
B A replica of the above gel was Western blotted and probed with anti-Rev antiserum (1/200 dilution).

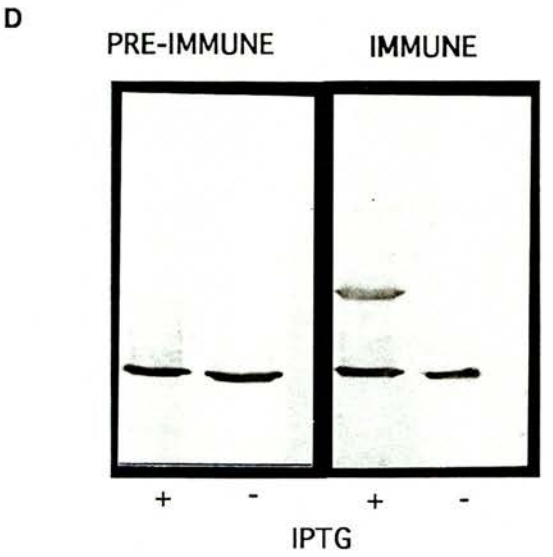
C Crude lysates of induced or uninduced (+ or - IPTG) cultures of the clones shown below were prepared by sonication. Proteins were resolved by SDS-PAGE on a 10% polyacrylamide gel. The gel was Western blotted and the fusion proteins detected by probing with anti-Rev antiserum (1/200 dilution).

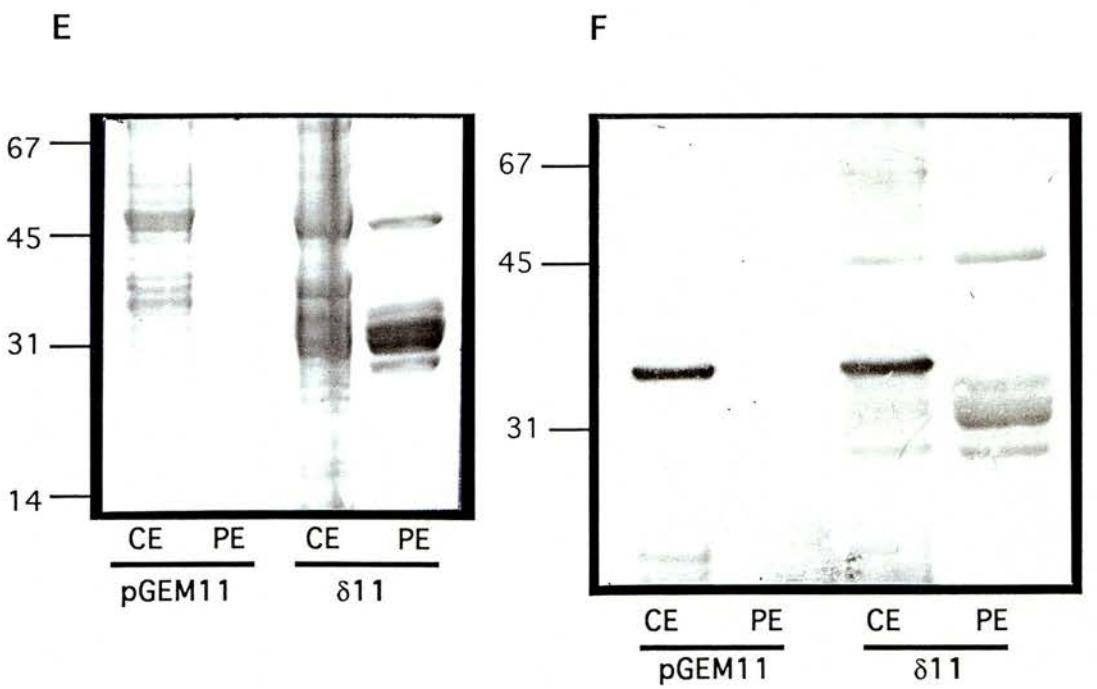
D As above, except only one pGEX2TRev transformant (δ11) was analysed. Blot was probed with pre-immune or immune serum (1/200 dilution) from rabbit immunised with synthetic Rev peptide (figure 4.2).

E Induced cultures of the pGEX2TRev transformant δ11, and of cells transformed with the pGEM11 plasmid, were harvested. Samples of crude extract (CE) and of glutathione-agarose bead purified eluate (PE) were resolved on a 10% SDS-PAGE gel. Proteins were visualised by staining with Coomassie blue.

F A replica of the above gel was Western blotted and probed with a rabbit anti-GST antiserum (1/100 dilution).


A
67
43
30
Molecular
Weight/kDa
GSTRev
GST
β2
β4
δ11
δ22
α6
α9
CLONE
B
67
43
30
Molecular
Weight/kDa
β2
β4
δ11
δ22
α6
α9
CLONE
C
Molecular
Weight/kDa
43
IPTG
--
+
--
+
--
+
δ11
δ22
α6
CLONE


D
PRE-IMMUNE
IMMUNE
+
-
+
-
IPTG


E
67
45
31
14
CE
PE
CE
PE
pGEM11
δ11
F
67
45
31
CE
PE
CE
PE
pGEM11
δ11

strong candidate for the fusion protein GSTRev.

To confirm that GSTRev is only produced by induced cells, duplicate cultures of two of the transformed clones, and one of the pGEX2T transformed clones, were generated. IPTG was added to one of each of the cultures, and the cells harvested after three hours. Anti-Rev antiserum was used to probe crude extracts of these cells after SDS-PAGE and Western blotting (Figure 4.4c and d). One positive band, with apparent molecular weight of 35-37kDa, is present in all extracts tested, and is believed to be due to non-specific recognition of a ubiquitous bacterial antigen. This is confirmed by figure 4.3d, which demonstrates that the pre-immune serum is also reactive with this protein. This band disappears on purification. However, it can be seen that a band with an approximate molecular weight of 46kDa appears only on induction of pGEX2TRev cultures (4.4c). The complete absence of this band from uninduced cultures suggests that the *tac* promoter is strongly repressed in the absence of IPTG, with little or no 'leaky' expression.

Finally, it was necessary to demonstrate that the candidate fusion protein was not expressed in bacterial cells as a general reaction to the presence of IPTG. Crude extracts and glutathione-purified samples of cells transformed with pGEX2TRev or with the unrelated plasmid pGEM11 and induced with IPTG for 4.5 hours were analysed by SDS-PAGE, and the proteins visualised by staining with Coomassie blue. From figure 4.4e it can be seen that no detectable protein is purified from the pGEM11 transformed bacteria by the addition of glutathione-agarose beads. This suggests that the 45-48kDa protein is specific to cells transformed with the plasmid pGEX2TRev. It is also apparent that native GST is not present in bacterial cells. This is supported by other authors (Frangioni and Neel, 1993).

Scanning densitometric analysis of Coomassie stained gels of purified GSTRev revealed that the 45-48kDa protein comprised between 10-18% of the total sample. The contaminating bands seen in figure 4.4a represent the largest fraction of purified protein. These impurities have been found consistently in purified samples, and are clearly positively selected for by the purification process. Similar contaminants have been noted in other GST fusion protein preparations (Tetzlaff *et al.*, 1992). Although it is possible that the contaminants are Rev binding proteins, co-purified with GSTRev, precedent suggests that instability of the fusion protein, resulting in proteolytic degradation, is the source of these contaminants (an alternative hypothesis is discussed in 4.8.3). The GST moiety is itself stable, hence degradation products would be selected for by the purification protocol. Removal of the

carboxyl terminal regions of the fusion protein containing the peptide antigen would render the degradation product invisible to the anti-Rev antiserum. The fastest migrating contaminating bands (27-28kDa) coincide with native GST and probably represent the complete removal of the Rev moiety from the recombinant protein. To demonstrate that the contaminating proteins were indeed GST-based, a replica of the gel shown in figure 4.4e was Western blotted and probed with a polyclonal anti-GST antiserum (a kind gift from Miss P. Carter, Department of Veterinary Pathology, University of Edinburgh). The resulting blot is illustrated in figure 4.3f. This antiserum recognises an approximately 36-38kDa antigen in whole cell extracts, in common with the anti-Rev antiserum. Pre-immune serum from this rabbit was also reactive with this band (P. Carter, personal communication): this strengthens the putative identification of this protein as a common bacterial antigen. In the lane corresponding to purified GSTRev, it is evident that all the proteins visible by Coomassie staining are also recognised by the anti-GST antiserum. No such blot positive material is detected in the pGEM11 cell extract, indicating that the pGEX2TRev plasmid must be the ultimate source of the contaminating proteins.

Fusion protein was therefore shown to be successfully produced by the transformed clones. One clone (δ11) was chosen for further study. Large scale preparations of fusion protein were produced by induction of 500ml cultures. Glutathione-agarose bound material was eluted with excess free glutathione.The efficiency of purification was difficult to assess, and it must be assumed that a proportion of the recombinant protein was lost during the purification process. It was therefore impossible to arrive at a figure for overall protein yields. However, typical yields of eluted protein were 12.5mg/L culture of native GST, and 3-4mg/L culture of GSTRev. There is therefore a 75-80% loss in yield of GSTRev compared to GST. Scanning densitometric analysis of Coomassie stained gels demonstrated that full-length GSTRev comprised between 10-18% of the total sample (0.3-0.75mg/L). This is probably a slight overestimate, due to the likely presence of contaminating proteins at levels below that required for detection by Coomassie staining. It was hoped that variation of growth and purification conditions might lead to both an explanation for, and an improvement in, the observed lower yield and stability of GSTRev in comparison with native GST.

4.5 Yield of GSTRev Fusion Protein

Although the yield of eluted fusion protein was relatively high, it was less than half that obtained with native GST. Reduction in yield could be a product of expression and/or purification. To attempt a distinction between these possibilities, crude extracts and purified eluates of induced pGEX2T and pGEX2TRev cultures were analysed by SDS-PAGE and Western blotting. Comparison of the Coomassie staining pattern of the crude extracts (Figure 4.5a) demonstrates that the band due to native GST is clearly visible as a higher proportion of total cellular protein than that due to GSTRev. Indeed, the band allocated to GSTRev within the crude extract may actually resolve into a doublet, with one of the bands representing a common bacterial protein also present in the GST crude extract. To examine the relative efficiencies of purification, a Western blot of this gel was probed with anti-GST antiserum (figure 4.5b). The relative signals of both GST and GSTRev are enhanced to an approximately similar extent within the purified eluate as compared to the crude extract. It seems unlikely, therefore, that gross differences in purification efficiency are responsible for the loss of yield of GSTRev.

Thus, although interpretation of the data was hazardous due to the difficulty in correctly determining the identity of the band corresponding to full length GSTRev in crude material, and by the fact that much of the purified GSTRev sample consists of degradation products of the fusion protein, it does appear that reduced protein expression is, at least in part, responsible for lower GSTRev yield. The growth characteristics of cultures expressing the fusion protein were studied in order to determine whether toxicity was a factor in this system.

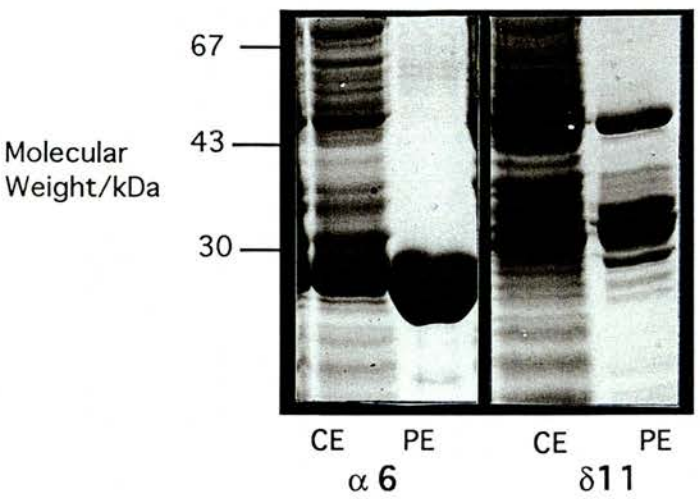
4.5.1 Growth Characteristics of pGEX Transformants

A time course study of the growth of both native and recombinant pGEX2T transformants was performed. Overnight cultures of both pGEX2T and pGEX2TRev were diluted into 200ml media and induced after one hour. The growth of cultures was monitored by OD_{600nm} readings for 5 hours. Uninduced cultures were also included in the time course study. Protein expression was confirmed by SDS-PAGE analysis of representative culture aliquots (data not shown). The results of a representative time course study are shown in

Figure 4.5: Analysis of Crude Extracts of Cells Expressing GSTRev

Medium scale (200ml) cultures of the clones α6 (expressing GST) and δ11 (expressing GSTRev) were induced with IPTG and cells harvested after 3 hours. Both crude extract (CE) and glutathione-agarose bead purified (PE) samples were analysed by resolution on a 10% SDS-PAGE gel, with protein visualisation by staining with Coomassie blue (A). A replica of this gel was blotted onto nitrocellulose, and probed with a polyclonal anti-GST antiserum (dilution 1/100) (B).

A


67
43
30
Molecular
Weight/kDa
CE
PE
CE
PE
α 6
δ11

B

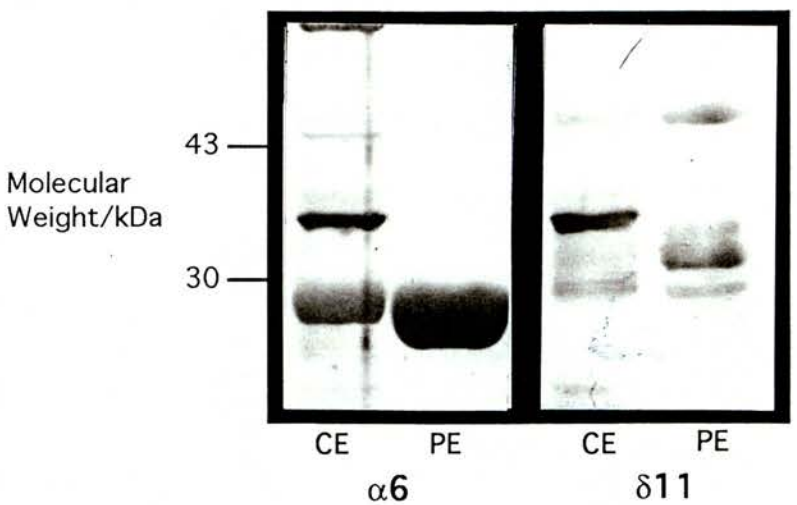

43
30
Molecular
Weight/kDa
CE
PE
CE
PE
α6
δ11

Figure 4.6a. Although it is clear that the dramatic toxic effect of Rev expression observed in transformed yeast cell cultures is absent in bacteria, there is a reduction in growth for both cultures on induction. The growth of pGEX2TRev transformants is affected more severely than that of the pGEX2T transformants. Although it is possible that this is a result of the greater physiological cellular stress due to the production of the larger fusion protein, the overall yield of GSTRev is less than that of native GST. It therefore seems possible that a degree of toxicity is present in this system. The lower yield of fusion protein might therefore be a result of the observed reduction in culture growth occasioned by GSTRev synthesis. However, it should be noted that after three hours (typical induction period for large scale preparations) there is an approximate 25% reduction in cell numbers between GSTRev and GST expressing cultures. This, by itself, is insufficient to explain the approximate 80% fall in yield of GSTRev compared to GST.

It would be expected that increasing the density of cell culture before induction would increase the yield of recombinant proteins with consequent toxic effects on the expressing cell. Conversely, if the toxic effect was minimal or absent, greatest yield would be promoted by induction at a lower density, allowing the cells to grow and divide vigorously in early log phase, while expressing protein. To investigate the effect of altering culture density before induction of GSTRev expression, cultures were grown to an OD_{600nm} of 0.3 or 0.55 before addition of IPTG. Growth was continued for three hours. Cells were harvested and the purified protein obtained from each culture analysed. From figure 4.6b, it can be seen that the yield of protein falls slightly on induction at the higher density (total yield ~1.4mg for lower density induction, ~1.2mg for higher density). This suggests that any toxic effect of Rev on bacterial cells is minimal in scope, and that the best yields of protein can be obtained by induction of expression at a lower cell density.

4.5.2 Fusion Protein Purification

Although the observed reduction in growth in cultures expressing GSTRev protein may account for reduced protein yield, it was possible that aspects of the purification procedure may also be a factor. In particular, partial insolubility of the fusion protein would reduce purification efficiency by sequestration of the insoluble fraction. To determine whether a significant proportion of GSTRev was insoluble, samples of supernatant and pellet obtained

Figure 4.6: Growth Characteristics of pGEX Transformants

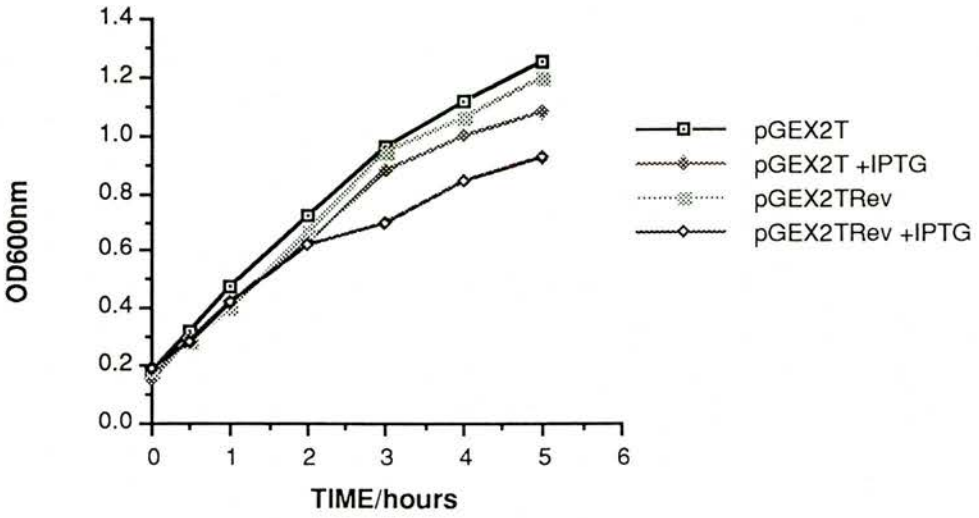
A: Growth of Colonies Transformed with pGEX2T and pGEX2TRev

Cultures derived from the selected δ11 pGEX2TRev clone were grown overnight and diluted 1/10 in fresh medium. After further growth to an OD_{600nm} of ~0.2, protein expression in one culture was induced with IPTG. Incubation was continued for 5 hours, with the culture OD_{600nm} recorded at regular intervals. Cultures derived from pGEX2T transformed cells were used as controls.

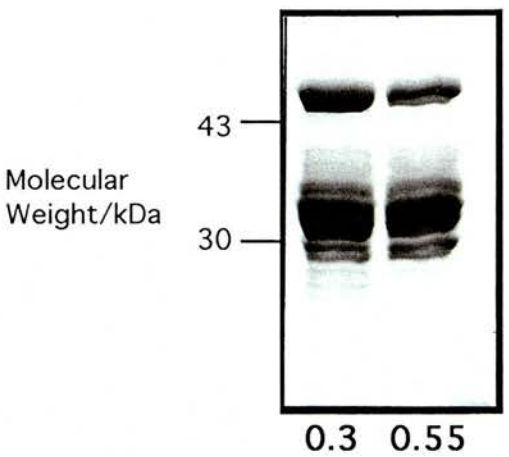
B: Effect of Cell Density on Expression of GSTRev

Medium scale cultures (200ml) of δ11 cells were grown and induced at the OD_{600nm} values shown on the diagram. Induction was continued for three hours, and the cells harvested. Recombinant proteins were purified and analysed on a 10% SDS-PAGE gel. Proteins were visualised by staining with Coomassie blue.

A


1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD600nm
0
1
2
3
4
5
6
TIME/hours
pGEX2T
pGEX2T +IPTG
pGEX2TRev
pGEX2TRev +IPTG

B


43
30
Molecular
Weight/kDa
0.3
0.55
Culture Density at Induction

after the centrifugation stage of protein preparation, immediately prior to affinity purification, were analysed for GSTRev content by SDS-PAGE and Western Blotting. On probing with anti-Rev antiserum it can be seen that a significant amount of the fusion protein is trapped within the pelleted debris after centrifugation (Figure 4.7:compare lanes 1 and 2). This may be due to one, or more probably a combination of several factors: incomplete solubility, incomplete cell lysis or interactions with cellular macromolecules. To determine whether the proportion of fusion protein within the soluble fraction and thus available for purification could be increased, the concentration of Triton X-100 was varied and the resulting supernatants compared (Figure 4.7 lanes 2-4). Triton X-100 is a non-ionic detergent included in the protocol to reduce interactions between proteins. It would appear that increasing the concentration of detergent from 1% to 1.5% (v/v) does increase the proportion of GSTRev within the supernatant. It should also be noted that the level of the ~36kDa protein additionally recognised by the Rev antiserum in crude extracts (section 4.4) increases concomitantly with GSTRev. As it had previously been shown that the ~36kDa protein is excluded by affinity purification, it was decided to use Triton X-100 at 1.5% in future preparations. This concentration of detergent should not alter the binding of fusion protein to glutathione-agarose beads (Frangioni and Neel, 1993).

4.6 Attempts to Increase the Purity of GSTRev Fusion Protein

It was previously shown that the contaminating proteins which comprise ~80% of GSTRev preparations were reactive with an anti-GST antiserum. It was concluded that product impurity was the result of fusion protein instability rather than contamination with heterogeneous bacterial proteins. Two approaches were taken in an attempt to increase fusion protein purity. Firstly, increasing stability would be expected to lower the concentration of contaminants. Secondly, varying purification conditions might allow separation of the full-length fusion protein from the contaminants.

4.6.1.1 Fusion Protein Stability: Purification

Instability of the full-length fusion protein may result in cleavage by proteases. This may occur within the bacterial cell during expression, and/or externally during purification.

Figure 4.7: Analysis of the Distribution of GSTRev between Soluble and Insoluble Fractions after Post-Lysis Centrifugation

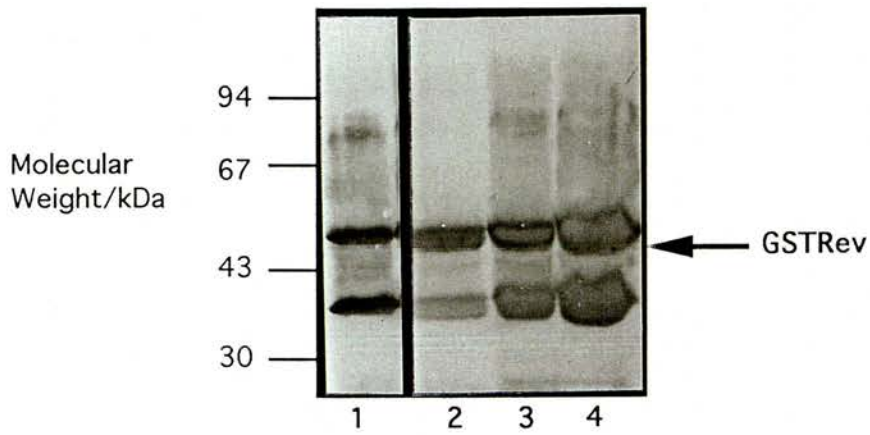
Cells induced to express GSTRev were harvested and lysed by sonication. After centrifugation at 10000g for five minutes, the supernatant and pellet were separated and a sample (0.25% of total volume) of each analysed for GSTRev content by SDS-PAGE and Western blotting. The blot was probed with anti-Rev peptide antiserum. Subsequently, the concentration of Triton X-100 added to the lysed cells prior to centrifugation was increased, and the GSTRev content of the resulting supernatants compared as above.

Lane 1: 1% Triton PELLET

Lane 2: 1% Triton SUPERNATANT

Lane 3: 1.25% Triton SUPERNATANT

Lane 4: 1.5% Triton SUPERNATANT


Molecular
Weight/kDa
94
67
43
30
1
2
3
4
GSTRev

Comparison of the profiles of GSTRev crude extract and purified eluate probed with anti-GST antiserum (figure 4.4f) demonstrates that all the final contaminants are present prior to purification. This suggests that degradation of the Rev polypeptide can occur within the cell, or during cell lysis. However, there is some suggestion of an increase in fragments with molecular weights between those of native GST and full length GSTRev in the purified eluate. Limited degradation of the remaining full length protein may occur during the purification process. The affect on stability of several alterations in the purification protocol was analysed. Addition of 0.25mM PMSF, a serine protease inhibitor, to cell lysates was routine. However, exclusion of PMSF did not alter the protein profile of GSTRev preparations. Similarly, inclusion of 50mM EDTA, an inhibitor of divalent cation dependent metalloproteases, in the lysis buffer, was without effect. Purification at 4°C had no visible effect, compared with routine preparation at room temperature. Similarly, altering the period of sonication from a minimum of 20 seconds to a maximum of 90 seconds, did not reduce the level of contaminants, suggesting that this procedure did not induce protein degradation. Thus, it was concluded that degradation occurs predominantly within the cell, or occurs rapidly on cell lysis, with the major enzymes involved not sensitive to the inhibitors used here, or to the effect of reduced temperature.

4.6.1.2 Fusion Protein Stability: Expression

It has been demonstrated that the host cell genotype can have a marked, and unpredictable effect on the expression of foreign genes in bacteria (Smith and Corcoran, 1990; Frorath *et al.*, 1991). In particular, the use of protease deficient strains may be beneficial (Buell *et al.*, 1985). To investigate the effect of bacterial genotype on expression of GSTRev, the plasmid pGEX2TRev was transformed into each of three different strains: DH5α, Y1090 (*lon* protease deficient), and BL21 (*lon* and *omp T* protease deficient). Protein expression was induced from small scale cultures of each strain, and purified protein analysed by SDS-PAGE (Figure 4.8a-d). Densitometric analysis was used to estimate the proportion of full-length protein obtained. It can be seen from figure 4.8 that contaminants of the same size range are present in all strains used. However, the proportion of full-length fusion protein does vary to some extent. In particular, the double protease deficient strain BL21 would appear to promote greatest protein stability. Approximately 20% of the protein eluate obtained from this strain is composed of full-length molecules, compared to 16% (JM101),

Figure 4.8: The Stability of GSTRev Fusion Protein

A-D: The Effect of Host Cell Genotype on Expression of GSTRev

The plasmid pGEX2TRev was transformed into cells of each of the strains of *E. coli* shown below. Small scale cultures (10ml) of each strain were induced with IPTG and cells harvested after 3 hours. Purified protein was analysed on 10% SDS-PAGE gels. Proteins were visualised by staining with Coomassie blue.

Figure A: JM101 clone: δ11

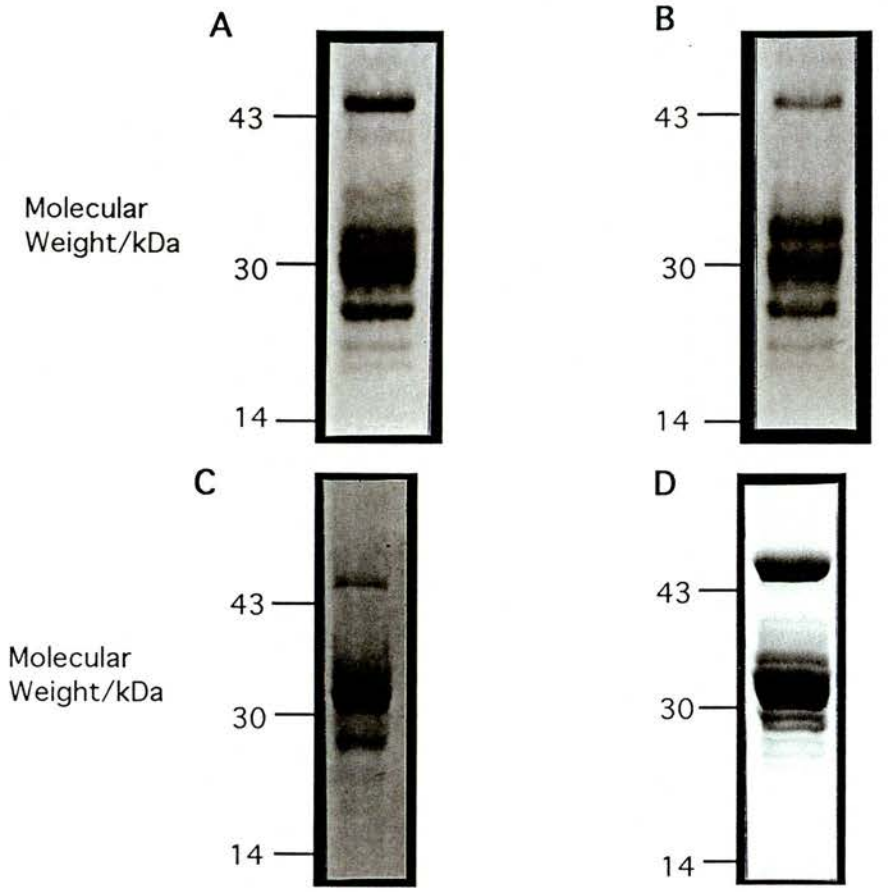
Figure B: DH5α clone: DH1

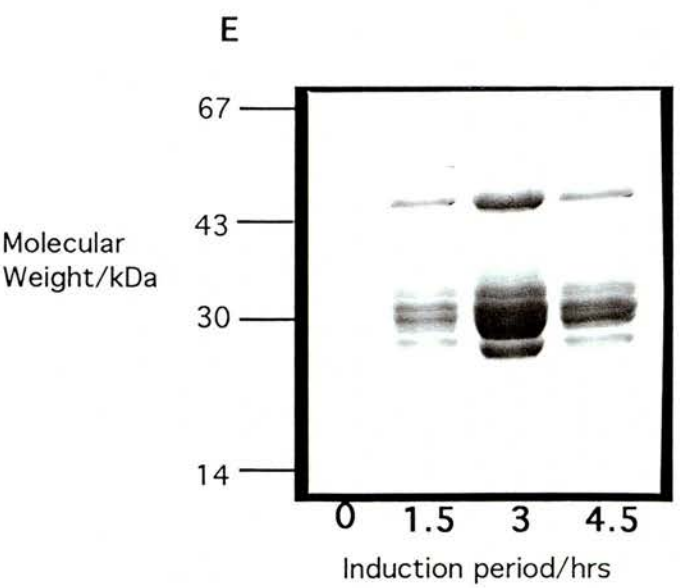
Figure C: Y1090 clone: Y7

Figure D: BL21 clone: B1

E: The Effect of Induction Period on the Expression of GSTRev

Medium scale cultures (200ml) of BL21 cells transformed with pGEX2TRev (clone B1) were induced with IPTG for 0-4.5 hours. Cells were harvested and purified protein analysed on a 10% SDS-PAGE gel. Proteins were visualised by staining with Coomassie blue.


A
B
C
D
Molecular Weight/kDa
43
30
14
Molecular Weight/kDa
43
30
14
43
30
14
43
30
14


E
Molecular Weight/kDa
67
43
30
14
0
1.5
3
4.5
Induction period/hrs

11% (Y1090) and 9% (DH5α). Although insufficient samples were analysed to provide statistically relevant data, the BL21 strain was chosen as the host for all subsequent protein production.

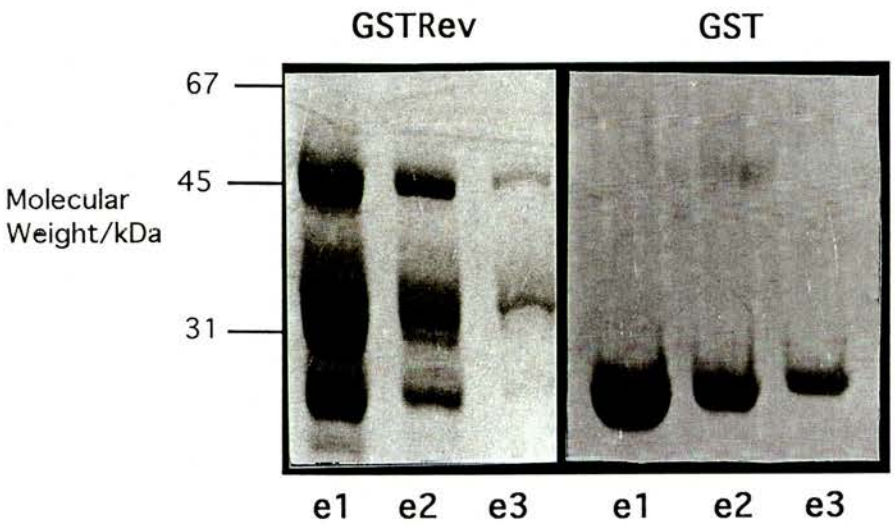
Reducing the induction period might be expected to lessen the effect of bacterial proteases (Ghosh *et al.*, 1995). Medium scale cultures (200ml) of BL21 transformants were induced for 1.5, 3 or 4.5 hours, and normal purification procedures carried out. Eluted proteins were resolved by SDS-PAGE and visualised with Coomassie blue (Figure 4.8e) and the proportion of full-length protein estimated by densitometry. There is no observed correlation between induction period and fusion protein stability. Indeed, reducing induction time from 3 to 1.5 hours is accompanied by a fall in the proportion of full-length protein from 20% to 16%. Variation in expression conditions was therefore not effective in increasing fusion protein stability.

4.6.2 Enhanced Fusion Protein Purity

During routine preparation of fusion protein, three sequential elution steps were undertaken. It was noted that there often appeared to be a decrease in the relative concentration of contaminating proteins with increasing elution number. SDS-PAGE analysis of sequential eluates of GSTRev and native GST from a typical preparation are illustrated by figure 4.9. The percentage of full-length fusion protein increases from 14% (first eluate) to 17% (second eluate) and 26% (final eluate). This phenomenon was observed in all studied preparations (although variation in the proportions of full length protein was recorded) and has been reported with other GST fusion proteins (N. Carey, PhD thesis, 1992). There is an accompanying decrease in the concentration of protein with sequential elution step, such that an average of ~75% of total protein is present in the first eluate. This affect of elution number on purity might be expected to be a function of the relative molecular weight of the full-length and contaminating proteins. Reduced glutathione might compete more efficiently with the smaller contaminants, therefore removing them from the agarose beads more rapidly than the larger, intact protein. The typical increase in full length protein purity with increasing elution number was not considered sufficient to outweigh the fall in concentration of product, and thus the first eluate alone was collected.

Figure 4.9: Analysis of Sequential Eluates of Purified GSTRev

Large scale cultures (500ml) of JM101 cells transformed with pGEX2TRev (δ11) and pGEX2T(α6) were induced with IPTG for three hours and the cells harvested. Expressed proteins were purified by the binding of glutathione-agarose beads to cell lysates. Bound material was recovered by elution from the beads in 50mM Tris.HCl pH8/ 10mM reduced glutathione for 5 minutes. Three sequential eluates (e1 e2 e3) were collected. Protein content of the eluates was examined on a 10% SDS-PAGE gel, with visualisation by Coomassie staining.


GSTRev
GST
67
45
31
Molecular
Weight/kDa
e1
e2
e3
e1
e2
e3

4.7 Proteolytic Cleavage of GSTRev

One reason for choosing the pGEX2T vector for the bacterial expression of Rev protein was the presence of a thrombin cleavage site in the adapter region between the Rev protein and the GST carrier. Moreover, a Factor Xa site was also included due to the use of the pOGS-based vectors as the source of the *rev* gene insert. It was hoped that cleavage of the Rev protein from the carrier could be followed by removal of the carrier by re-adsorption with glutathione-agarose beads, resulting in a pure Rev product. Fusion protein eluates were incubated with either protease at concentration ratios of 0.2%, 1% and 4% (w/w). After six hours, the reactions were boiled to terminate cleavage, and the products analysed by SDS-PAGE and Western blotting (Figure 4.10). Visualisation by Coomassie staining (4.10a), reveals the removal of the 48kD band corresponding to the full-length fusion protein and its replacement with a band of enhanced intensity at ~28kD, corresponding to native GST, in the protease treated samples. This demonstrates that cleavage with both enzymes proceeds efficiently, with digestion to completion at protease concentrations of 0.2% for Factor Xa and 1% for thrombin. It is also observed that the contaminating, intermediate bands are removed by proteolysis. These proteins must therefore contain both thrombin and Factor Xa recognition sites, further confirming the status of these contaminants as degradation products of the fusion protein.

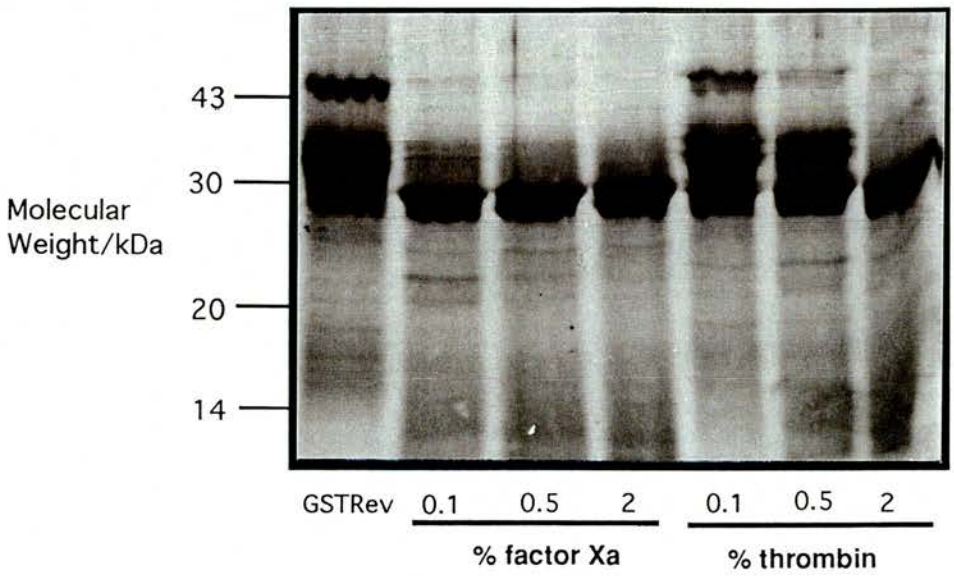
There are no obvious candidate bands for released Rev protein revealed by Coomassie staining. However, blotting with anti-Rev antiserum (4.10b) reveals the presence of two bands, migrating closely together at ~20kDa, in all the cleaved fractions. These are thought to represent the full-length Rev protein. The more rapidly migrating of the two bands is present predominantly in the Factor Xa cleaved samples, with the more slowly migrating band predominant in the thrombin cleaved samples. Cleavage with thrombin is expected to give rise to a polypeptide six amino acid residues (sequence: GSIEGR) larger than that released by Factor Xa cleavage, due to the positioning of the respective proteolysis sites. One explanation for the existence of the double band is therefore that the minor band is the product of non-specific protease activity at the heterologous recognition site. It is also possible that contamination of each enzyme source with small concentrations of the other enzyme not removed during purification from plasma might account for these results.

With increasing protease concentration, it is seen that the bands allocated to full-

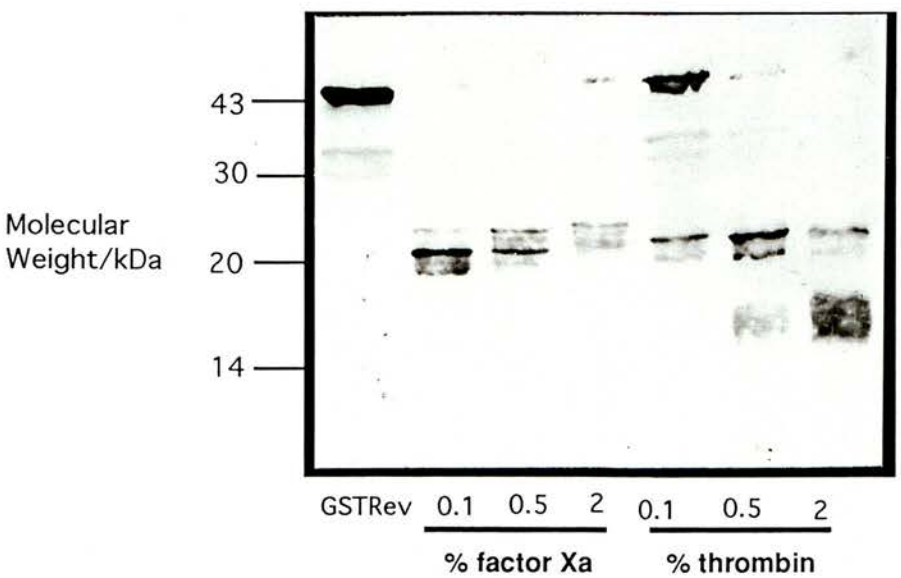
Figure 4.10: Protease Digestion of GSTRev

GSTRev (~800µg) was incubated with either thrombin or factor Xa at the concentrations shown, for six hours at 25°C. Samples were analysed by SDS-PAGE on a 10-20% polyacrylamide gradient gel. Proteins were visualised by staining with Coomassie Blue (A), or by Western blotting and probing with an anti-rev antiserum (B).

A


Molecular Weight/kDa
43
30
20
14
GSTRev
0.1
0.5
2
0.1
0.5
2
% factor Xa
% thrombin

B


Molecular Weight/kDa
43
30
20
14
GSTRev
0.1
0.5
2
0.1
0.5
2
% factor Xa
% thrombin

length Rev decrease in intensity. This suggests that secondary cleavage sites for both enzymes are present within the Rev moiety of the fusion protein. An antiserum positive smear is present at ~16kDa when thrombin was used at high concentrations (4.10b); this is not present on cleavage with Factor Xa. The proteases thus appear to act at different secondary sites. The number and position of such sites is difficult to determine. Factor Xa cleavage does not produce additional blot positive bands. Thus, cleavage at a single site may remove the carboxy-terminal ~50 residues containing the peptide recognised by the antiserum, rendering the major fragment invisible to blot detection, with the blot positive fragment too small for SDS-PAGE detection, or cleavage at multiple sites may result in near complete proteolytic degradation. Thrombin cleavage may remove 3-4 kDa at either terminus to preserve the blot positive status of the major 16kDa fragment. The broad smear obtained suggests limited exolytic activity uncharacteristic of a site-specific protease, and may be due to the high concentration of enzyme or enhanced activity of contaminating proteases on the partially cleaved protein. The proliferation of Rev fragments produced by the promiscuous action of the proteases on both the full-length and partially degraded fusion proteins would result in small numbers of each fragment size. This would explain the failure to allocate a single Coomassie-stained band to the Rev polypeptide. Reduction of incubation time from six hours to one hour did not prevent secondary cleavage events (data not shown).

In summary, cleavage of the Rev protein from the GST carrier proceeded efficiently. However, the presence of different substrate molecules and the non-specific activity of the proteases led to a plethora of cleaved fragments and an inability to isolate a full-length Rev polypeptide. It was therefore decided that cleavage of the fusion protein would not be used to produce Rev protein for functional studies.

4.8 Discussion

This chapter describes the construction of a vector to direct high level expression of recombinant EV1 Rev protein as a fusion to the glutathione-S-transferase enzyme of *Schistosoma japonicum*. Analysis of expressed and purified protein demonstrated that successful expression had been achieved. Several strategies to improve the yield and purity of the recombinant protein have been attempted, with at least some success.

4.8.1 Protein Yield

The yield of eluted GSTRev was typically 3-4 mg/L culture. Although this figure is in the middle of the range of yields recorded by the authors of this system (Smith and Johnson, 1988), it is nevertheless less than that obtained for native GST (12.5mg/L). Lew and co-workers (1991) have produced purified GST/Protein A at ~50mg/L. One rationale for the use of a fusion system is the presence of both *cis-* and *trans-* acting factors known to give rise to a good yield and quality of product. Variation in yield on insertion of a foreign gene must therefore be due to the effect of that gene and its products on the carrier system. Induction of GSTRev protein synthesis has been shown to retard the growth of host cells. However, it would appear that the reduction in growth is not sufficient to explain fully the reduced yield of GSTRev. The extent and form of possible Rev-mediated toxicity in bacterial cells must remain speculation. Increasing culture density before induction of expression did not increase the yield of fusion protein as might be expected. The product of the *env* gene of MVV has been shown to exert a severe toxic effect in bacteria (N. Carey, PhD Thesis 1992). This might implicate the amino-terminal region of Rev, which is shared with the Env glycoprotein, in potential toxicity. However, the stretches of hydrophobicity thought to be responsible for the toxic effect of viral glycoproteins expressed in bacteria (Sisk et *al.*, 1992) are not present within the Env/Rev common region.

The alteration of growth conditions in ways other than attempted in this chapter might also influence protein expression. Authors reporting successful production of GST fusion proteins have used a hundred-fold variation in the concentration of the inducing agent, IPTG, from 0.1mM (Smith and Johnson, 1988; this thesis) to 10mM (Frosch *et al.*, 1991). Higher concentrations of IPTG within the culture may ensure maximal induction of the *tac* promoter, by 'mopping up' the product of the *lacI*^{q} gene. Although this might be expected to increase expression by boosting transcription, it could have the opposite effect if mRNA or protein toxicity was a factor. Pressure of time did not allow investigation into this aspect of expression.

Reduced expression of GSTRev might also be due to factors other than protein toxicity. Transcriptional or translational efficiency and mRNA stability might also influence the level of GSTRev. Translational efficiency, in the form of codon usage, will be discussed below. It seems probable that a combination of factors is responsible for differences in recombinant protein yields, with each protein possessing unique characteristics.

Although experimental data suggested that purification was not a limiting step with respect to GSTRev yield, small, incremental increase in yield might have been possible to obtain by alterations to the purification protocol. Affinity purification requires a soluble substrate. A degree of insolubility promoted by the foreign protein would reduce the pool of molecules available for binding to the glutathione-agarose beads. Although insolubility is often associated with a high hydrophobic content and large molecular size, it has been found to be a common problem with the pGEX system (Frangioni and Neel, 1993). A proportion of the recombinant GSTRev is present in the discarded pellet after post-sonication centrifugation (4.5.2). This could be the result of partial insolubility of the fusion protein, but it is also likely that much of the pelleted material is trapped inside intact cells or sequestered within macromolecular complexes, perhaps bound to cellular RNA. The increase in the level of GSTRev within the supernatant correlating with increased concentration of the detergent Triton X-100, supports the latter explanation. There is therefore no evidence to suggest that GSTRev is not readily soluble. Increasing the concentration of Triton X-100 did not give rise to a consistent increase in final protein yield.

The protein binding capacity of the glutathione-agarose beads is estimated at >8mg/ml (Sigma). Frangioni and Neel (1993), have suggested that the capacity of the beads decreases sharply with increasing substrate size, by a factor larger than would be expected for constant binding on a molar basis. Thus, the 47kDa GSTRev protein would saturate the beads at a significantly lower concentration than the 27.5kDa native GST. Increasing the number of beads used in the purification process might lead to increased yield. The elution of protein from the beads may also be a limiting factor. As described above (4.6.2) smaller proteins may elute more readily from the beads than larger ones. An increase in the period of elution might therefore increase the yield of GSTRev with respect to GST. Other measures which might aid elution include altering the concentration of free glutathione and the use of nonionic detergent in the elution buffer (Frangioni and Neel, 1993). The concentration of glutathione in the eluting buffer has been reported as high as 25mM (Tetzlaff *et al.*, 1992). Increasing elution efficiency might be expected to improve yield. However, approximately 75% of all eluted GSTRev was recovered in the first of three eluates, suggesting that the protocol used was relatively efficient, and that elution was not a limiting step.

It has therefore proved impossible to determine exactly which aspect of expression and/or purification is responsible for variation in yield between native GST and GSTRev. It is

probable that several factors are involved. The instability of the full length fusion protein is undoubtedly linked to reduced yield; cleaved residues are lost from the final purified material. For further elucidation, a more complete examination of the various stages of protein production would be required; this was not considered an appropriate subject for further study.

4.8.2 Protein Purity

Scanning densitometric analysis of electrophoresed samples of purified GSTRev demonstrated that the eluted material consisted of 10-20% full length product. Contaminating protein bands were identified consistently at the same positions, and were found to be reactive with an anti-GST antiserum. This suggested that these contaminants were stable intermediate products of proteolysis of the full length fusion protein. Proteolysis of proteins expressed in bacteria has been widely reported (Goldberg and St. John, 1976; Nakano *et al.*, 1994). The product of the fusion between GST and the *Babesia bovis* merozoite protein Bb-1, for example, is cleaved almost to completion (Tetzlaff *et al.*, 1992). Both primary and higher order protein structural features are likely to determine susceptibility to proteolytic degradation. In the present study, the GST moiety is not cleaved, which suggests that it and the Rev polypeptide form independent folding domains. This may conversely be beneficial, as it may promote functionality of the fusion protein. The major contaminating proteins have apparent molecular weights in the range 28-34kDa, which represents the carrier molecule in addition to approximately 0-6.5kDa of Rev. This would suggest that the largest of the contaminants contains the 60 amino-terminal residues of Rev (based on an average amino acid residue molecular weight of 110Da). Degradation of the fusion protein was shown not to occur significantly during purification. Degradation might therefore occur within the cell or during cell lysis. Two strategies were employed in an attempt to reduce the proportion of fusion protein affected by proteolysis. Firstly, the period of induction of expression was reduced. This was not found to lead to reduced degradation. It may be that a shorter period of induction than the 1.5 hours used in this study might be beneficial. Secondly, the effect of bacterial host genotype on expression was investigated. Studies using a β-galactosidase somatomedin-C (a human serum protein) chimaera, have shown that a higher yield and longer protein half life are obtained in the protease deficient *E. coli* strain SG936 compared to other

strains (Buell *et al.*, 1985). Variation in GSTRev fusion protein stability between host cells was noted, although the differences were not large. The strain BL21 was found to promote the greatest stability. Two protease genes, *lon* and *ompT*, are absent in this strain. The Y1090 strain is also *lon* deficient, which would appear to implicate the *ompT* product in the proteolysis of GSTRev. Bonds between successive basic residues are especially susceptible to OmpT cleavage (Sugimura and Higashi, 1988); such bonds are common within the Rev polypeptide. Proteolysis by a membrane associated enzyme occurs predominantly during cell lysis (Sugimura and Higashi, 1988). Uncharacterised differences between bacterial strains with respect to protease activity cannot, however, be discounted. It may be possible to further increase stability and yield of GSTRev by use of other bacterial strains. A second *ompT* deficient strain, AD202, is also associated with enhanced stability of certain GST fusion proteins (Nakano *et al.*, 1994). Cells with a mutation of a heat shock regulatory gene *htpR* have been shown to promote stability of expressed proteins (Baker *et al.*, 1984). The effect of bacterial proteases may also be countered by reducing the incubation temperature for expression. Growth of cells expressing a GST/MVV Env fusion protein at 25°C has been shown to reduce the level of contaminants (P. Carter, Department of Veterinary Pathology, University of Edinburgh, personal communication). The relative abundance of full length forms of heterologous GST fusions has been reported to be increased by incubation at a lower temperature and for a shorter period. Such conditions do not universally bring success, however (Ghosh *et al.*, 1995).

A different approach would be to employ the recently described vector pETGEXCT (Sharrocks, 1994). This vector, a hybrid containing elements of both the pET and pGEX systems, allows foreign polypeptides to be expressed as fusions at either terminus of GST. Fusion of the fungal transcriptional activator protein QUTA to the amino terminus of GST has been shown to reduce protein proteolysis associated with fusion to the carboxyl terminus (Sharrocks, 1994).

4.8.3 Codon Utilisation

Although instability of the fusion protein was initially assumed to be the reason for the presence of contaminating proteins in purified preparations, experimentation did not provide conclusive support for this hypothesis. In particular, reduction in induction period did not

reduce the level of contamination. An alternative explanation was possible. The rate of utilisation of synonymous codons is non-random, and varies between different organisms. *E. coli* exhibits a strong bias against several codons; for example, four of the six codons specifying arginine are rarely used. Two of these, AGA and AGG, are the least frequently observed codons in the E. *coli* genome (Zhang *et al.*, 1991). Thus, the presence of rare bacterial codons in genes of non-bacterial origin might affect translational efficiency: difficulty in translation of foreign genes containing such codons has been predicted by Konigsberg and Godson (1983). Experimentally, reduced expression of full length eukaryotic protein in transformed *E. coli* has been associated with increasing the percentage content of AGA and AGG codons (Brinkmann *et al.*, 1989). However, Varenne and co-workers recorded that consecutiveness, rather than total content, of rare codons was critical (1989). The number of consecutive rare codons may be directly proportional to the repressive effect on correct translation (Rosenberg *et al.*, 1993): this may vary between individual genes and according to the intramolecular position of the rare codons within each gene.

The explanation for the inhibitory effects of rare codons on translation lies in the fact that the frequency of codon occurrence reflects the cellular abundance of cognate tRNA species (Ikemura, 1981). Thus, although isolated rare codons can be decoded efficiently, the presence of consecutive codons causes a pause in translation with the first codon bound in the ribosomal peptidyl-tRNA site due to a shortage of tRNA required for the downstream codon bound at the amino acyl-tRNA site. This could lead to uncoupling of the mRNA from the ribosomal complex resulting in premature translational termination. Alternatively, ribosomal slippage may recruit a more abundant tRNA species, resulting in a frameshift event. Two consecutive AGG codons induce 50% frameshifting into the +1 frame in the rat interferon α1 gene (Spanjaard and van Duin, 1988). A high error rate was also recorded in the presence of tandem AGA codons. Such events would be manifest by reduced yield of full-length protein and the presence of aberrant or truncated polypeptide molecules. If the truncated molecules adopted a stable secondary structure, they might be expected to avoid proteolytic cleavage which is the usual fate of abnormal proteins (Goldberg and St. John, 1979).

The *E. coli* frequency profile of codons present in pGEX2TRev is illustrated in Figure 4.11. The eight most rare codons (as defined in Zhang *et al.*, 1991) comprise 19 of the 153 *rev* (plus linker region) codons (12.3%), but only 14/224 (6.2%) of the GST codons. There is a particular bias toward the arginine codons AGA and AGG in the *rev* gene; these comprise

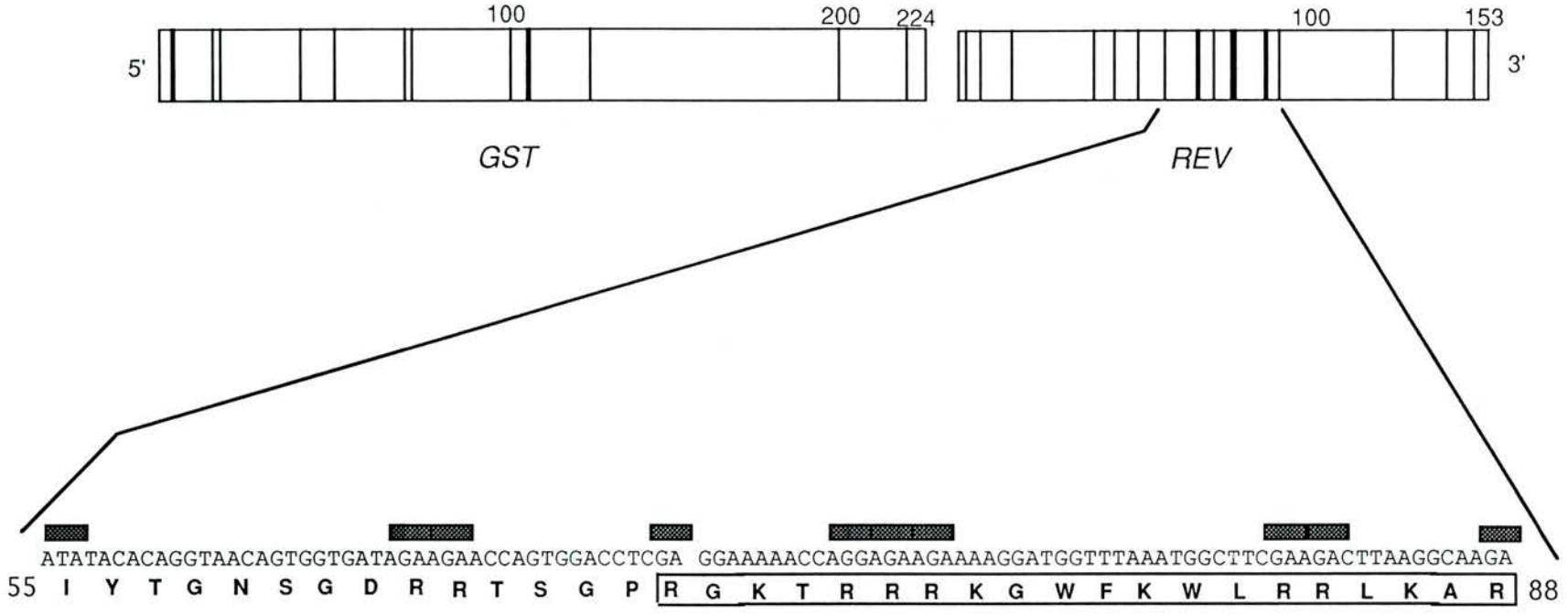
Figure 4.11: Distribution of Rare Bacterial Codons in pGEX2TRev

The genes *gst* and *rev* are illustrated. The position of the eight codons most rarely used by *Eschericia coli* bacteria is marked by a vertical line. Rare codons are defined as follows (frequency of codon occurrence as defined in Zhang *et al.*, 1991).

AGG	Arg
AGA	Arg
AUA	Ile
CUA	Leu
CGA	Arg
CGG	Arg
CCC	Pro
UCG	Ser

Numbers indicate codon position within each gene. The highlighted region (*rev* codons 55-88) has a high density of rare codons. The DNA sequence from this region is shown. Rare codons are indicated as follows: ▩

The amino acid sequence encoded by this region is shown in bold. The boxed region indicates the predicted basic domain of Rev (Sargan *et al.*, 1991).


100
200
224
5'
GST
100
153
3'
REV
ATATACACAGGTAACAGTGGTGATAGAAGAACCAGTGGACCTCGA GGAAAAACCAGGAGAAGAAAAGGATGGTTTAAATGGCTTCGAAGACTTAAGGCAAGA
55 I Y T G N S G D R R T S G P R G K T R R R K G W F K W L R R L K A R 88

7.8% of the total (12/153) compared to 0.9% in *gst* (2/224). The region between residues 69-81 within *rev* is particularly rich in rare codons (6/13), largely due to the preponderance of arginine residues. No such region is present within GST. Thus the distribution of rare codons within pGEX2TRev correlates with the high level expression of intact GST and the presence of shortened Rev molecules. The tandem AGA codons at *rev* codon positions 63/64 and at 74/75 may induce ribosomal frameshifting or disengagement. Frameshift into the -1 register would halt translation at one of several termination codons: there are no such codons in the +1 register. A full-length protein could thus be generated by +1 frameshifting; however, this would not be recognised by the anti-Rev peptide antiserum. Although electrophoretic analysis of GSTRev suggested that the largest of the contaminating proteins would contain approximately 60 residues of Rev, and the other contaminants considerably fewer, it is possible that anomalous migration of products truncated at positions 63/64 or 74/75 could account for this difference. Limited degradation of the truncated protein could also occur, reducing molecular weight.

Translation of consecutive rare codons is also linked to reduced bacterial growth and viability (Brinkmann *et al.*, 1989). The minor tRNAs specific for arginine are required for chromosomal replication: sequestration of these molecules in polysomal complexes would inhibit cell division. Expression of those bacterial genes specifying rare codons might also be repressed. This provides a possible explanation for the reduced growth of pGEX2TRev transformed cells on IPTG induction.

Removal of the rare codons by mutagenesis to synonymous codons found more frequently in bacteria would determine whether this hypothesis was valid. The number of such codons found in *rev* would, however, make this a difficult task. An alternative approach would be to co-transform host cells with a vector directing high level expression of the appropriate $tRNA^{arg}$ species. Co-expression of the *argU* gene, encoding the bacterial $tRNA_{AGA/AGG}$, resulted in high level expression of the full length 28kDa protein of barley yellow mosaic virus RNA2 (Shenk *et al.*, 1995): a minor truncated product was observed in the absence of *argU*.

The effect of rare codons could also explain the reduced yields of GSTRev compared to native GST. Bogerd and co-workers (1991) have reported an approximately two-fold increase in yield of a GST/HTLV-1 Rex fusion protein on substitution of two rare arginine codons for ones more efficiently utilised by bacteria.

A different approach to improving the purity of GSTRev would be to isolate the full-length molecules from the contaminants by size exclusion chromatography. The relatively small differences in size involved meant that such an approach would require considerable manipulation of matrix size and conditions for success. Time did not allow for an investigation into the practicalities of such a strategy.

4.8.4 Cleavage of the Fusion Protein

Thrombin and restriction factor Xa are serine proteases with highly specific target sequences. Although both cleave arginyl and/or lysyl bonds, the environment required for efficient cleavage has been incompletely elucidated: hence, prediction of cleavage activity is imprecise. Thrombin cleaves selectively at only two of 181 R/K-X bonds in fibrinogen (Blomback *et al.*, 1967). However, low efficiency cleavage may occur at a large number of such bonds (Chang, 1985). This phenomenon has also been observed to occur with factor Xa digestion (Nagai and Thogersen, 1987). An internal site (63-YLGRSAE-69) within the HIV-1 Rev molecule is susceptible to partial secondary digestion (Jensen *et al.*, 1995). Cleavage at secondary sites has been reported to coincide with protein instability (Riggs, 1992). Thus the suggested instability of the GSTRev protein may contribute towards the observed multiple cleavage events on treatment with both proteases. As a result of the inability to allocate actual bands on Coomassie stained gels to the products of such cleavage events, it is impossible to determine the number and identity of secondary sites. Examination of the amino acid sequence of Rev enables some conclusions to be drawn about possible secondary sites, however. The basic cleavage sites for the two enzymes are (from Chang, 1985; Nagai and Thogersen, 1987):

Thrombin : X--R--Y, with X preferred P or G, and Y is often G, V, D, I, C, or R.

Factor Xa : X--R--Y, where X is usually G, and Y is often I or T, never P.

In both cases, lysine may replace the central arginine at certain sites. Other surrounding residues may also be influential. The closest matching potential protease site throughout GSTRev is at position 135-137 of Rev, with the sequence G--R--D. Cleavage at this position would release an eleven residue fragment, removing 1.63kDa from the Rev molecule, and

leaving the antiserum positive peptide intact. Minor reactivity at this site might explain the double band of ~20kDa observed on protease digestion. Following initial digest the susceptibility of the protein to further cleavage may increase, possibly due to alteration of the higher order structure. The data concerning thrombin digestion implies cleavage 3-4kDa from either terminal region: a thrombin secondary cleavage at the G--R--D sequence might initiate less specific events resulting in removal of this residue mass. Positions 68-70, P--R--G, of Rev provide a second close match to the thrombin consensus sequence. Cleavage at this position would effectively halve the Rev molecule, producing two fragments of approximately 9.5kDa. This does not fit the data obtained for thrombin cleavage.

It must be emphasised that prediction of cleavage sites is inexact. As an example, internal thrombin cleavage within the Maltose Binding Protein/Poliovirus 2C fusion occurs in the absence of a predictably susceptible arginine bond (Rodriguez and Carrasco, 1995). More data concerning the sizes of the products of cleavage would be required before a more accurate analysis of potential sites would be possible.

4.8.5 GSTRev for Functional Assays

The aim of the work of this chapter was to produce Rev protein for *in vitro* functional studies. Unfortunately, pure Rev protein could not be obtained, due to the instability of the protein and its susceptibility to secondary cleavage by proteases. However, it is possible that the intact fusion protein, GSTRev, could be functionally equivalent to native Rev. Several groups have reported successful functional studies using proteins attached to an expression carrier. A GST/Influenza A virus NS1 protein fusion has been shown to bind NS1 target RNA in *in vitro* binding assays (Qian *et al.*, 1994). Similarly, in the period since the work of this chapter was begun, several groups have reported successful expression and functional characterisation of HTLV-1 Rex, HIV-1 Rev and MVV strain 1514 Rev proteins. Binding activity of a GST/HIV-1 Rev fusion has been shown to be indistinguishable from that of wild type Rev (Jensen *et al.*, 1995) (Discussed in more detail in chapter 6). However, it should be noted that correct activity of the expressed protein is not always observed. Gearing and co-workers (1989) demonstrated that a GST/leukaemia inhibitory factor (LIF) fusion was non functional, due to conformational restraint. Correct activity was released on protease cleavage of the fusion protein. Thus, the major factor determining fusion protein functionality is likely to be the

higher order structure. The possible susceptibility of the Rev but not the GST portion of the fusion protein to proteolytic degradation suggests that the two polypeptides form independent folding domains. This would be likely to promote Rev functionality. This suggestion, along with the evidence of other groups for the activity of intact fusion proteins, led to the decision to use GSTRev as the source of Rev activity for functional assays. The main potential problem with this approach would be the presence of the contaminating, truncated GSTRev molecules. Whereas those contaminants which consisted of the GST polypeptide alone, or with a small Rev content, would not be expected to interfere with the functional assays, the larger contaminants might be problematic. Data from SDS-PAGE gel analysis of the fusion protein suggests that the largest of the contaminants should contain up to 60 residues of Rev; thus the predicted RNA binding domain of Rev (section 3.2) would not be present in any of the contaminants. However, the actual size of the contaminants could be significantly different, due to anomalous migration in the SDS-PAGE system. The potential binding or interfering activity of truncated products of GSTRev would have to be taken into consideration when interpreting results from such binding assays.

4.9 Summary

A vector to direct the inducible expression of MVV Rev as a fusion to the enzyme GST was constructed. A protein of the predicted molecular weight, and reactive with an antiserum raised against a synthetic Rev-derived peptide, was shown to be specifically produced by bacterial cells transformed with this vector. The total yield of protein, although less than that of native GST, was found to be within expected limits. Fusion protein stability was found to be problematic. Approximately 80-90% of purified protein consisted of truncated fragments of the fusion protein. Several different approaches were taken in order to improve yield and purity of product, with some success. In particular, the use of host cells with protease deficient phenotype, was found have some benefit. Protease cleavage of the fusion protein, to release the Rev moiety, resulted in secondary cleavage events within the Rev polypeptide. It was decided that the fusion protein would be used for Rev functional studies in its uncleaved form.

CHAPTER FIVE

IN VITRO STUDIES OF GSTREV RNA BINDING

5.1 Introduction

RNA molecules in biological systems are rarely, if ever, present in naked form, but instead are complexed with a range of RNA-binding proteins. Many RNA-binding proteins mediate specific, discriminatory interactions. These interactions are of significance for the fate of intracellular RNA. The processing, nucleocytoplasmic export and translation of pre-mRNAs, for example, are dependent on events mediated by specific RNA binding proteins. The structural determinants of RNA/protein interactions are poorly understood. In part, this is a consequence of the structural versatility of RNA molecules. RNA molecules can adopt a range of higher order structures, which are likely to form elements recognised by proteins. 'Stem' structures formed by base pairing between regions of sequence complementarity are commonly recognised elements, as are internal deformations in such regions caused by non-base paired nucleotides.

The protein motifs involved in RNA binding are also poorly characterised. However, three distinct classes are presently recognised. The conservation of these motifs suggests that common protein structural elements may be used for target recognition. The RNA recognition motif (RRM, also RNP, ribonucleoprotein motif) is the most commonly occurring of these classes, and is recognised in over 200 distinct proteins (Bandziulis *et al.*, 1989). Many of these are involved in RNA processing, and include 5' cap-binding proteins and spliceosome components (for a review of the RRM, see Nagai *et al.*, 1995 and references therein). Two conserved domains of 6-8 residues within a less conserved ~80 residue region constitute this motif. Certain nucleolar and hnRNP proteins contain a second RNA recognition motif, characterised by arginine/glycine/glycine repeats (Kiledjian and Dreyfuss, 1992). A diverse group of molecules constitute the third class of RNA-binding proteins, those with a distinct basic residue binding motif containing a preponderance of arginines. Bacteriophage anti-terminator N proteins, ribosomal proteins and RNA viral capsid proteins are included within this group (Lazinski *et al.*, 1989). The arginine-rich binding domain is characterised on the basis of a high arginine/lysine content over a stretch of 10-15 amino acid residues, with little additional sequence similarities. Electrostatic interactions between the positively charged arginine residues and the negatively charged phospho-diester RNA backbone are probably involved in initiating contact between molecules. It seems likely that non-arginine residues are important in making discriminatory contacts with RNA targets to give these proteins their

individual specificities (Lazinski *et al.*, 1989). Proteins containing an arginine-rich binding motif are able to make specific contacts with a variety of RNA secondary structures.

RNA/protein interactions play a critical role in the life cycle of those viruses, such as the retroviruses, which possess an RNA genome. HIV-1 encodes three proteins with RNA-binding activities, Gag, Tat and Rev. The RNA binding of both Tat and Rev is dependent on arginine-rich motifs (Dingwall *et al.*, 1989; Malim *et al.*, 1989b). Isolated peptides containing these motifs are competent to mediate target binding with similar characteristics to the full length protein (Weeks *et al.*, 1990; Kjems *et al.*, 1992). However, recent evidence suggests that, despite the similarities in the binding domains, Tat and Rev RNA binding is mediated by different structural features (Calnan *et al.*, 1991; Tan *et al.*, 1993; Tan and Frankel, 1995).

Both of these regulatory proteins recognise their specific target sequences in the context of complex RNA secondary structure (Muesing *et al.*, 1987; Malim *et al.*, 1989c; Malim *et al.*, 1990). This recognition is critical for virus viability. The Tat recognition element, TAR, consists of a single stem-loop, with recognition mediated by a trinucleotide bulge at the stem-loop intersection (Dingwall *et al.*, 1990).The RNA target for HIV-1 Rev, the RRE, is more complex, consisting of a long stem of duplex RNA and five stem-loops. It has been predicted to contain 234nt (Malim *et al.*, 1989c), although more recent analysis has suggested that 351nt are required for full function (Kimura and Ohyama, 1994; Mann *et al.*, 1994). A core high affinity binding site within the RRE consists of ~13 non-contiguous nucleotides (Heaphy *et al.*, 1991) with a helix-internal loop-helix structure (Bartel *et al.*, 1991).

The experimental determination of Rev and Tat RNA interactions was achieved by the use of *in vitro* binding assays. The substrates for these assays were recombinant proteins and *in vitro* transcribed RNA. Sequences corresponding to the predicted RNA targets were cloned into vectors containing promoters for bacteriophage DNA dependent RNA polymerases, and transcribed in the presence of radioactively labelled ribonucleotide triphosphates. Protein/RNA interactions can be observed by electrophoresis through non-denaturing polyacrylamide gels with subsequent autoradiographic visualisation. Complex formation is indicated by a 'shift' in the position of the band due to the labelled substrate as a consequence of reduced electrophoretic mobility; hence the term 'gel retardation assay'. This assay may be quantified by densitometric analysis of the distribution of free and retarded probe signals. Alternatively, protein/RNA complexes may be detected by filtration through a nitrocellulose filter in conditions which favour the retention of complexes but the filtration of

non-complexed substrate. Filter binding assays are scored by the counting of radioactive substrate bound to the nitrocellulose after filtration.

The presence of a highly basic region with 7/22 arginine residues (3.2.2) within the MVV EV1 Rev protein was suggestive of an RNA binding capability similar to that of HIV-1 Rev. Computer predictions of secondary structure were used to define an RNA region within the EV1 envelope gene, close to the encoded surface glycoprotein/transmembrane protein (SU/TM) cleavage site, which may adopt a stable secondary structure with similarities to the HIV RRE. In order to investigate possible interactions between EV1 Rev and this putative RNA element, this region was cloned into vector plasmids and used to generate radioactively labelled RNA by run-off transcription. Binding reactions were carried out using partially purified GSTRev fusion protein (chapter 4). Potential complex formation between protein and RNA was monitored by both gel retardation and filter binding assays.

5.2 A Potential Rev Responsive Element within the EV1 Envelope Gene

The Rev responsive elements of the primate lentiviruses are positioned immediately downstream of the boundary between the coding region for the SU and TM glycoproteins (Malim *et al.*, 1989c; Le *et al.*, 1990). Computer simulation of the secondary structure of RNA from this region of the Maedi Visna virus EV1 was used in an attempt to locate a similar element. Figure 5.1 illustrates a 176 nucleotide RNA secondary structure from this region with a predicted free energy of formation of -70.5kcal/mol (prediction programme based on Zuker, 1989). Minor variations in formation energy were recorded by the use of slightly different sequence elements; however, the basic structure observed in figure 5.1 was conserved. The secondary structure begins at nucleotide 7995 and terminates at 8170. The element is therefore located nine bases downstream of the predicted SU/TM boundary (Sargan *et al.*, 1991): this is remarkably consistent with the location of the HIV-1 RRE (Malim *et al.*, 1989c). Although there is no primary sequence homology with the RREs of any of the primate lentiviruses, the secondary structures contain conserved elements. In particular, these regions are characterised by a main helix of double stranded RNA enclosing several stem-loop structures. The EV1 element contains three stem loops, two of which are linked by a duplex region within the 5' section of the structure: this arrangement is similar to that of the HIV-1 RRE (see figure 1.6). The high affinity Rev binding element of the HIV-1 RRE is situated

Figure 5.1: The Predicted Rev Responsive Element of MVV EV1

Computer prediction of the RNA secondary structure within the transmembrane glycoprotein coding sequence of EV1 *env*. Position 1 corresponds to nucleotide 7986 of the published EV1 genomic sequence (Sargan *et al.*, 1991). The secondary structural element begins at position 9 (7995) and terminates at position 186 (8170). The ΔG_f for this structure is -70.5kcal/mol. Computer prediction made using the UWGCG (version 8) programme 'FOLDRNA' and represented by the programme 'SQUIGGLES'.

140
130
150
160
170
180
190
200
120
50
40
30
20
10
110
100
60
90
80
70

within the base of one of these stem-loops. Thus, by analogy a possible site for EV1 Rev may be found within these structures. The presence of only one additional stem-loop within the EV1 element in contrast to the three found in the HIV-1 RRE is intriguing, given the functional redundancy of these domains within HIV-1 (Holland *et al.*, 1990). This further suggests that the Rev binding site may be located within the two linked stem-loops.

A report on secondary structure elements within the *env* gene of the Cork strain of CAEV appeared to confirm the above prediction for the ovine/caprine lentivirus RRE (Saltarelli *et al.*, 1990). This report also predicted the structure of the putative RRE for the 1514 MVV strain. All three putative RREs are situated in identical genomic locations and are 176nt in length. The relationship between these elements will be investigated in more detail in chapter 6.

5.3 Generation of an RNA Substrate for Functional Assays

In order to determine whether EV1 Rev was an RNA binding protein with specificity for the putative response element within the *env* gene, a series of functional assays were carried out. These involved detection of ribonucleoprotein complexes formed by protein:RNA interactions. The protein substrate for these assays was the fusion protein GSTRev. Transcripts generated by *in vitro* transcription of the putative RRE by insertion into appropriate plasmid vectors were to serve as the RNA substrates.

5.3.1 *In Vitro* Transcription

RNA transcripts can be generated from cloned DNA sequences by the use of DNA dependent RNA polymerases derived from certain bacteriophages. The *Eschericia coli* bacteriophages T3 and T7, and the *Salmonella typhimurium* SP6 encode polymerases with several characteristics suitable for *in vitro* transcription. These include the ability to transcribe any sequence placed downstream of the 23bp consensus promoters; the requirement for a simple reaction buffer with no additional transcription factors (Melton *et al.*, 1984), and high specificity for transcription from the cognate promoter (Butler and Chamberlin, 1982). Moreover, these polymerases demonstrate high specificity with respect to the DNA strand transcribed: this latter property is of particular importance in the current context. T7 or SP6

polymerase transcription from a linearised template with a 5' protruding or blunt terminus gives rise to less than 0.2% transcripts derived from the non-coding strand (Cox *et al.*, 1984; Schenborn and Mierendorf, 1985). Several commonly used vectors encode RNA polymerase promoters adjoining the multiple cloning site. Thus, RNA can be obtained by run-off transcription from a plasmid linearised with an appropriate restriction enzyme. The amount of extraneous sequence derived from the vector can thus be kept to a minimum. With all four ribonucleotide triphosphates (rNTPs) present in the reaction mixture at saturating concentrations (>50μM) a yield of 10-20 copies of RNA per template can be obtained (Melton *et al.*, 1984). For product detection, a radioactively labelled rNTP is included in the reaction. The concentration of this substrate will therefore be lower than that of the other rNTPs, and this may result in a lower yield of product. Moreover, disengagement of the polymerase from the template at sites where the limiting nucleotide is required may give rise to a proportion of incompletely synthesised molecules (Melton *et al.*, 1984).

5.3.2 Transcription from a pTZ18R-Based Vector

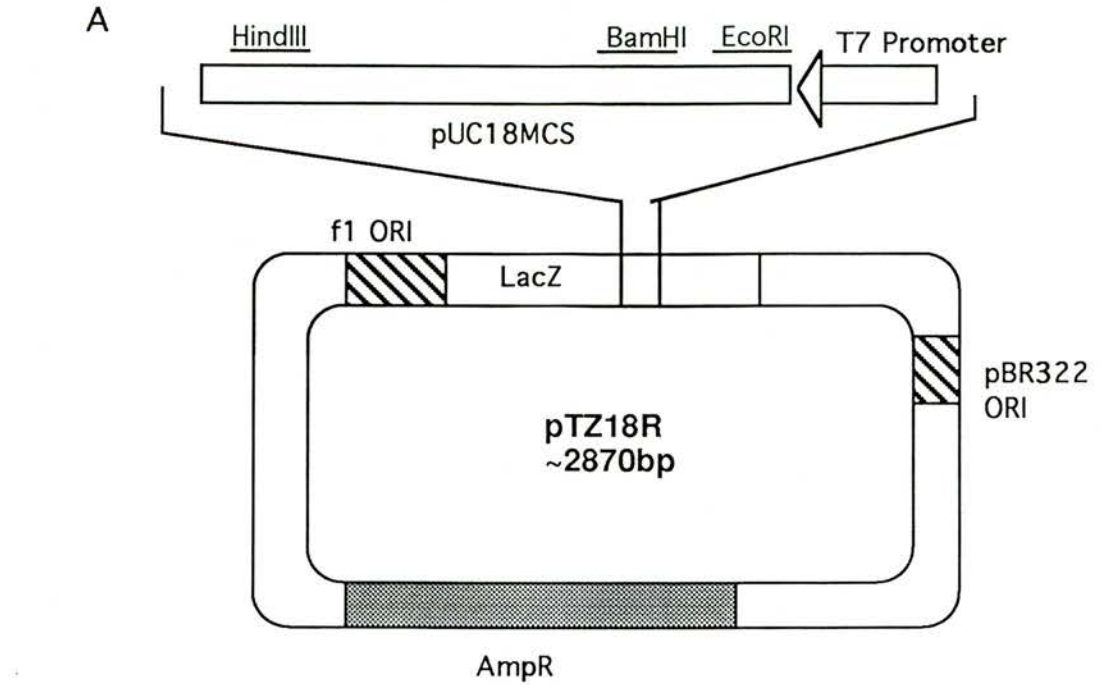
A template for the synthesis of the putative EV1 RRE was available in the form of a vector derived from pTZ18R and containing the 5' region of the *env* gene, encoding the amino-terminal portion of the transmembrane glycoprotein, gp46 (a kind gift from C. Cousens). This plasmid (pCCR6) contained an insert consisting of the gp46 bases 1-462 (EV1 bases 7986-8449), which was cloned into pTZ18R via a digestion site for the restriction enzyme EcoRI. The vector pTZ18R is depicted in figure 5.2a. A single T7 promoter is located at the junction of the multiple cloning site within three base pairs of the EcoRI site, and thus can be used to direct transcription from the insert containing only three bases of vector-derived sequence. This plasmid was available in two forms, with the insert present in both possible orientations, allowing synthesis of both the plus strand (template pCCR6(+): sense RRE) and negative strand (pCCR6(-): antisense RRE) which would serve as a control. The vectors were linearised with the restriction enzyme BamHI for the synthesis of sense RRE, and with HindIII for the synthesis of antisense RRE. Different restriction sites were used to avoid confusion: but were not functionally necessary, as the insert was cloned in a different orientation in each plasmid. Vectors were obtained from large scale preparations using the 'Qiagen' system (2.10). RNase was excluded from the protocol, in an effort to avoid possible

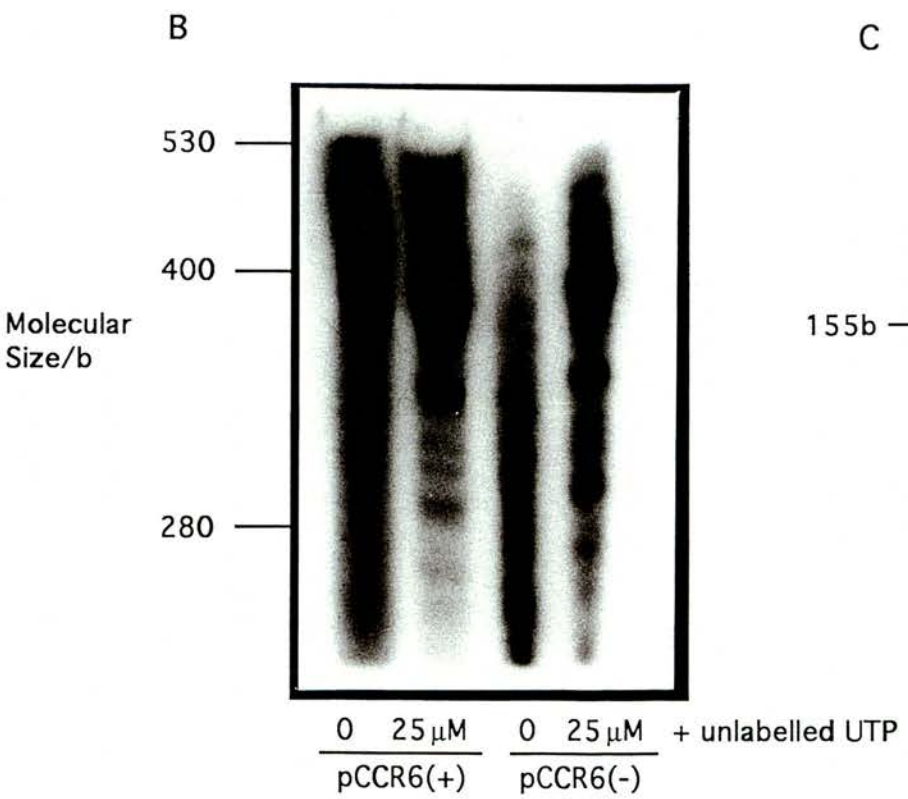
Figure 5.2: Transcription from the pTZ18R Vector, pCCR6

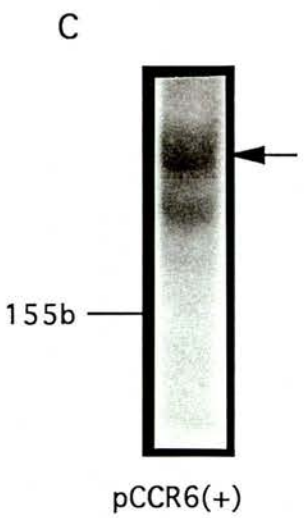
A The parental vector pTZ18R. This plasmid contains a multiple cloning site (MCS). The position of restriction sites used here are shown. The MCS is situated within the *lacZ* coding sequence, which allows production of β-galactosidase fusion proteins from inserted sequence. A T7 RNA polymerase promoter directed toward the MCS, allows the production of RNA from a sequence insert. The plasmid contains two origins of DNA replication (ORI) and a β-lactamase ampicillin resistance gene for selection.

B *In vitro* transcription from linearised, full insert length pCCR6 plasmids. Transcription was carried out according to standard procedures with 5μl (50μCi) α-^{32}P UTP in the presence or absence of 25μM unlabelled UTP as indicated. After incubation for one hour, an aliquot was used to determine the percentage of label incorporated. Approximately 10000cpm of product were analysed by electrophoresis through a 6% denaturing (8M urea) polyacrylamide gel, and products visualised by autoradiography.

C *In vitro* transcription of AvaI linearised pCCR6(+). Transcription carried out as above, using 25μM unlabelled UTP. Arrow indicates potential full length transcript (~200nt).


A
HindIII
BamHI
EcoRI
T7 Promoter
pUC18MCS
f1 ORI
LacZ
pBR322
ORI
pTZ18R
~2870bp
AmpR


B
530
400
280
Molecular
Size/b
0 25μM
pCCR6(+)
0 25μM
pCCR6(-)
+ unlabelled UTP


C
155b
pCCR6(+)

contamination at later stages. Plasmid was digested with the appropriate restriction enzyme, and digested plasmid isolated by excision from a 1% low gelling temperature agarose gel (2.8). The digested plasmid was purified with silica (2.5.3) and phenol extracted, before resuspension in TE at a concentration of ~1mg/ml. This procedure was deemed necessary in order to prevent contamination with undigested plasmid, which could direct the transcription of vector-length RNA molecules and greatly reduce the efficiency of insert transcription (Melton *et al.*, 1984). Transcription was carried out using a commercial kit obtained from AMS Biotechnology (2.18.3). Ten units of the RNase inhibitor RNasin (Promega) were included in each reaction. The radiolabelled rNTP chosen was α^{32}P-UTP, which had a specific activity of 400μCi/mmol and was present in the reaction mixture at a concentration of 6.25μM. The reaction products were visualised by electrophoresis through a 6% denaturing polyacrylamide gel with subsequent autoradiography (2.18.4.2) and the efficiency of the reaction gauged by precipitation of RNA with trichloroacetate (2.18.4.1). The transcripts obtained by this procedure are illustrated in figure 5.2b. It is clear that the proportion of full-length transcript (465nt) produced is low: a number of bands with reduced molecular size are present. Additionally, the indistinct radioactive signal is suggestive of a problem with degradation, especially with the antisense RRE (lane 3). There is essentially no full-length antisense RNA. The efficiency of the transcription reaction was also low, and somewhat variable: the percentage of α^{32}P UTP incorporated into RNA was on average 24.2 +/- 14.2% for sense RNA, and 19.7 +/- 4.2% for the antisense RNA (mean +/- standard deviation, for three independent reactions). In an attempt to increase the proportion of full-length molecules, unlabelled UTP was included in the initial reaction at a concentration of 25μM. Although this would result in a considerable reduction in the specific activity of the RNA probe, the benefit in terms of yield might make this acceptable. From figure 5.2b it can be seen that although there is some decrease in the proportion of smaller sized truncated fragments associated with the presence of cold UTP, the yield of full-length product remains low.

Ling and co-workers (1989) have reported that the yield of radiolabelled full-length product may be considerably reduced due to abortive initiation of transcription, if the limiting nucleotide is present within the first 8-12 bases from the transcriptional start point. The first twenty bases transcribed from pCCR6(+) and pCCR6(-) are as follows:

pCCR6(+): **CU UAU GU** GGG **CAU** AG GG**U U**G

pCCR6(-): **CU UAA U U**CUU **UGU**GC A**U**CCC

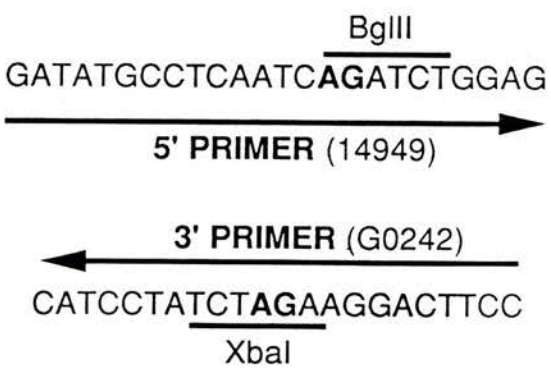
where the initial trinucleotide CUU is derived from the pTZ18R vector, and positions 4-7 (AUGU) in the sense transcript are derived from the primer used to clone this region (C.Cousens, PhD thesis, 1993). The preponderance of uridines within the antisense transcript (9/20) may explain the low incorporation achieved, due to a high rate of abortive initiation. The alternative use of cytidine triphosphate as the label was contra-indicated by the presence of this base in the first position after transcription initiation. Moreover, it was clear that even when initiation proceeded normally, there was a high level of subsequent termination. The vector context and insert sequence have been reported to affect the success of *in vitro* transcription with bacteriophage polymerases (Melton *et al.*, 1984). In particular, the coincidental presence of a region with homology to the poorly defined polymerase terminating sequences within the insert may induce transcript truncation. The length of the full-length transcripts (466b: 463 derived from insert and 3 from the vector) was in excess of that required for transcription of the predicted RRE, so it was decided to utilise a unique AvaI restriction site within the insert (position 219) to reduce the transcript size. It was hoped that this would encourage the production of full-length transcripts, by removing regions which may induce truncation. An AvaI site within the multiple cloning site of pTZ18R (position 197) is located downstream of the T7 promoter/insert, such that digestion resulted in removal of the 3' section of the insert with no affect on the template. pCCR6(+) was digested with AvaI and used as template for transcription of the sense strand. Unlabelled UTP (25μM) was included in this reaction. The result of this experiment is depicted in figure 5.2c. The putative full-length product (222 bases, arrow) comprises approximately 60% of the total, a considerable improvement on the previous results. The efficiency of transcription, as judged by TCA precipitation, remained low, however. A major contaminating fragment, of approximately 200 bases, is present. Intact HIV-1 RRE transcripts have been reported to migrate as two bands under native gel conditions (Benko *et al.*, 1990), presumably due to the ability to form distinct conformations. However, it was also possible that this species was a degradation or truncation product. Possible competition for Rev binding between these two fragments meant that this was not a suitable reagent for functional assays. Isolation of the full-length fragment by denaturing gel electrophoresis with subsequent elution of the RNA was

found to be an inefficient method of purification. The percentage of label incorporated into RNA remained low with this template, as would be expected if there was significant abortive termination.

It was thus decided to proceed by utilising an alternative vector for RRE transcription, and to alter the composition of the insert by including different flanking sequences. It was hoped that in this way the problems of truncation might be avoided.

5.3.3 Transcription from a pGEM-based Vector

A region including the putative RRE was obtained by Polymerase Chain Reaction (PCR) using EV1 DNA extracted from infected ovine choroid plexus cells as template, in order to study Rev function in transient transfection assays (see chapter 6). It was decided that this fragment would be a suitable template for *in vitro* transcription. The primers used for PCR incorporated selected restriction sites to allow sub-cloning of the product into the transient transfection assay vector, pOX100 (chapter 6). However, these sites could also be utilised to ligate the insert into the plasmid, pGEM11Zf(+) (obtained from Promega). This vector contains dual, opposing bacteriophage polymerase promoters, for the T7 and SP6 enzymes, flanking the multiple cloning site (figure 5.3a). The primers used for amplification were as follows (nucleotides shown in **bold** represent changes from the genomic sequence designed to incorporate restriction sites):



The 5' primer (14949) is predicted to hybridise with EV1 genomic positions 7920-7943, and the 3' primer (G0242) with 8207-8228. The amplified DNA fragment should thus have a size of 309 bp. PCR was carried out under standard conditions (2.4.3) and the result is

Figure 5.3: Cloning of Putative RRE into the pGEM11Zf(+) Vector

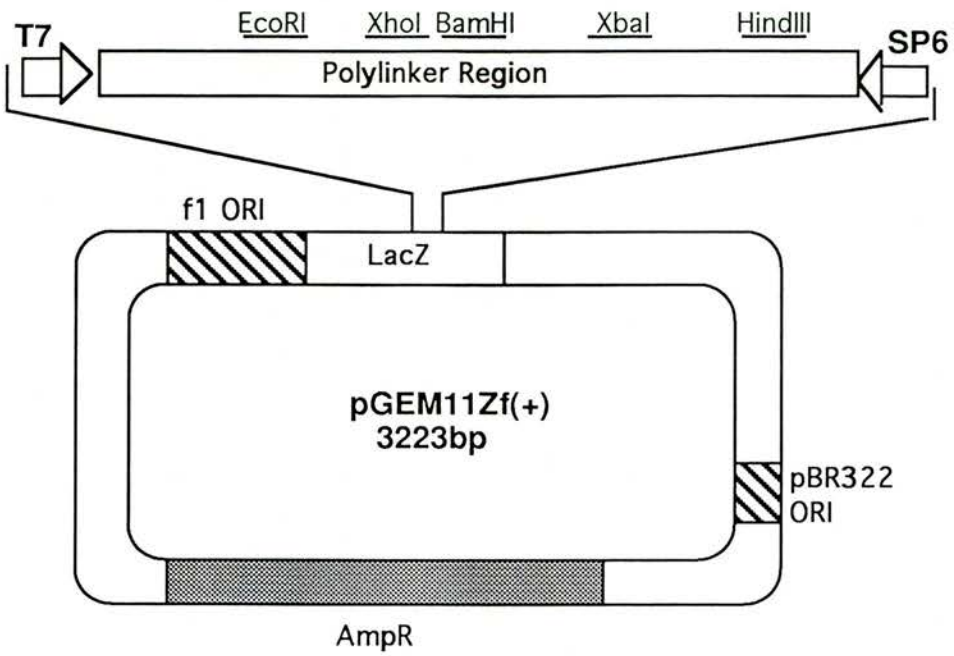
A The parental vector pGEM11Zf(+). This plasmid contains similar elements to pTZ18R (figure 5.2). The multiple cloning site (or polylinker region) is larger, and several restriction sites are indicated. Flanking the polylinker are two, opposing RNA polymerase promoters, for the T7 and SP6 enzymes.

B PCR amplification of DNA fragments containing the putative EV1 RRE. PCR was performed using viral genomic DNA as template, and oligonucleotide primers as described in the main text. A: primers 14949 and 14948 (see chapter 6) B: primers 14949 and G0242. The product of reaction B was termed RRE.9/2

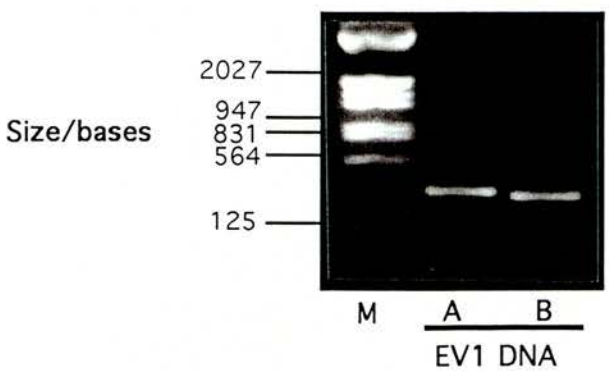
C Nucleotide sequence of the insert RRE.9/2. The fragment obtained by PCR (B) was purified and ligated into the pGEM11 vector. Sequence was obtained using double stranded sequencing reactions and the standard M13 reverse sequencing primer, which hybridises to pGEM11. Sequence flanked by the unique restriction sites used for subcloning is shown. The position of the start of the sequencing region for the transmembrane glycoprotein, gp46 (TM) is indicated. The region predicted to form secondary structure is marked (RRE start and end). Base changes from the published EV1 sequence (Sargan *et al.*, 1991) are highlighted in **bold**.

D Map of the plasmid pGEM11/RRE. The arrow indicates insert orientation. The region of secondary structure within the insert is stippled. The linearised templates for RNA synthesis are also shown. Each template shows the utilised RNA polymerase promoter, restriction site and the number of transcribed bases derived from each region.

A


T7
EcoRI
XhoI
BamHI
XbaI
HindIII
SP6
Polylinker Region
f1 ORI
LacZ
pGEM11Zf(+)
3223bp
pBR322
ORI
AmpR

B


Size/bases
2027
947
831
564
125
M
A
B
EV1 DNA

C

BglII

GGA TCT GGA GAA AAA TAG AAG AAA TGC TTT TAA GAA

TM start RRE start

AGA AAG AAA GAA GAG GGG CAT AGG GTT GGT TAT AGT

GCT CGC TAT CAT GGC AAT AAT AGC TGC TGC AGG AGC

TGG TCT CGG TGT TGC AAA TGC CGT GCA GCA GTC CTA

TAC AAG GAC GGC TGT CCA GTC TCC TGC TAA CGC AAC

TGC TCA GCA GAA TGT GTT AGA AGC AAC TTA TGC CAT

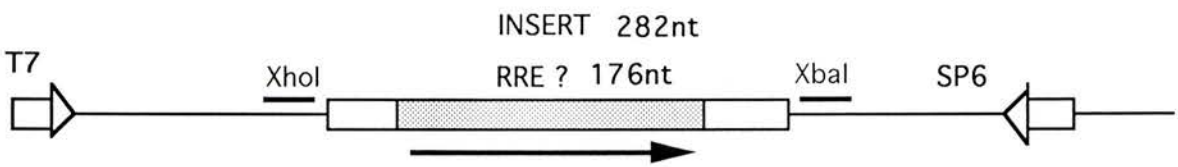
RRE end

GGT GCA TGT AGC CAA AGG AGT AAG AAT ATT AGA GGC

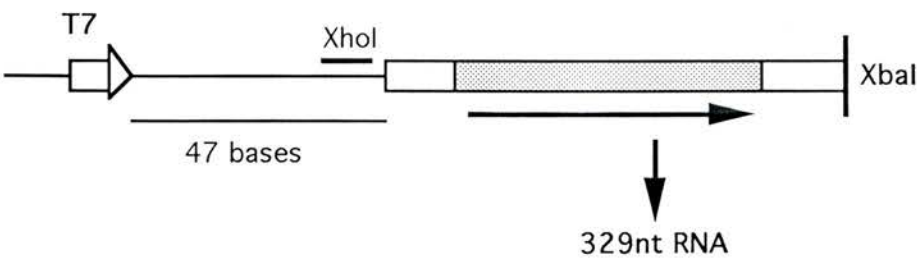
AAG AGT TGC CCG CGT GGA AGT CCT TCT AGA

XbaI

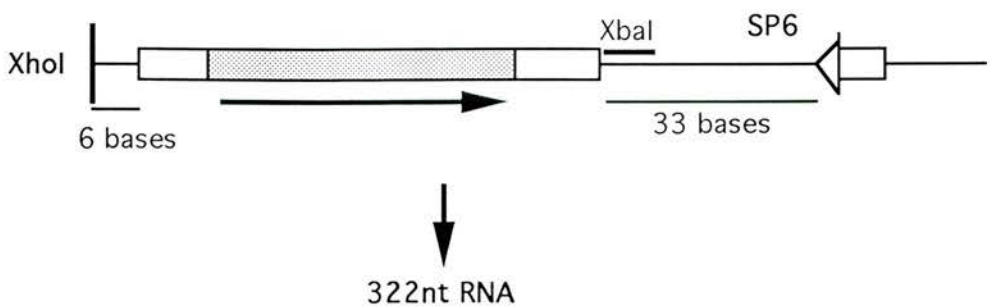
D



tT7 - sense RNA



tSP6 - antisense RNA



illustrated by figure 5.3b. The fragment generated (RRE.9/2) was of the expected size, and was cloned in the first instance into the pCR™II vector using the TA cloning kit (In Vitrogen; 2.7.1). A small scale DNA preparation of this plasmid was produced, and ~2μg used for restriction digest with BglII and XbaI. The excised fragment was located by agarose gel electrophoresis, and purified by hot phenol extraction (2.5.2). An aliquot of the vector pGEM11Zf(+) was digested with the enzymes BamHI and XbaI, and dephosphorylated with CIP (2.7.1). The enzymes BamHI and BglII produce compatible protruding ends, which can religate to produce a sequence susceptible to digestion with neither enzyme. This feature was used to subclone the RRE fragment directly into pGEM11Zf(+), which does not contain a BglII recognition site. Vector and insert were ligated and transformed into DH5α bacteria. Transformants containing a plasmid with the expected restriction digest characteristics were used to generate a large scale plasmid preparation. The recombinant plasmid, designated pGEM11/RRE, contains EV1 sequences 7935-8216, and was sequenced using the M13 reverse primer, which hybridises to the parental vector at positions 128-144. The sequence obtained is shown in figure 5.3c. There are four base changes between the cloned insert and the corresponding published sequence (which forms the insert in pCCR6). These all occur within the putative RRE. Prediction of secondary structure based on these changes suggests a more stable structure (ΔG_f -78.9kcal/mol) than that found in the original sequence. This perhaps suggests that the changes are authentic sequence variants and not artefacts of the amplification and cloning process.

To generate templates for *in vitro* transcription, the plasmid pGEM11/RRE was digested with appropriate restriction enzymes (figure 5.3d). XbaI was used to generate template for sense RNA, using the T7 promoter (linearised template referred to as tT7). A unique XhoI site situated 6bp upstream of the 5' end of the insert was used to generate antisense RNA by utilising the SP6 promoter (template tSP6). A possible problem with the use of pGEM11 as host vector for transcription is the relatively large amount of extraneous sequence separating the promoter sites from the insert (47 bases for the T7 promoter and 40 for the SP6 promoter). These regions might participate in the formation of secondary structure and interfere with the native RNA form of the insert. To investigate this possibility, computer predictions of the structure of the entire transcript generated by transcription from both promoters was performed. From figure 5.4 it can be seen that additional elements of secondary structure are present in the sense pGEM11/RRE transcript. However, the putative

Figure 5.4: Predicted Secondary Structure of RNA Transcribed from the pGEM11/RRE Vectors

Computer predictions as for figure 5.1.

A Sense RNA, derived from tT7

B Antisense RNA, derived from tSP6

10
20
310
300
320
30
290
40
50
60
280
70
110
100
270
80
120
90
260
130
140
250
240
230
220
150
210
160
170
200
180
190

60
50
70
40
80
30
90
20
100
110
130
140
120
230
220
240
250
260
270
280
150
310
290
210
160
170
180
200
190
300

RRE is intact and is identical to that predicted for this region alone. The presence of extraneous stem-loop structures gives the whole element a stability of -101.5kcal/mol., and may thus conversely be beneficial by stabilising the putative RRE. The possibility of non-specific binding to Rev mediated by the extraneous sequences must be considered when interpreting results of experiments using this RNA. The structure of the antisense transcript contains a region with some similarity to the sense element; in particular the presence of a central helix surrounding three stem-loops, two of which are linked. However, there are important differences in terms of the size and positioning of substructures, and the primary sequence is divergent. Certain regions of double stranded RNA stems within the antisense probe form mirror images of the sense probe, although these are not extensive. The high degree of secondary structure demonstrated by the antisense transcript suggests that it is a suitable control to study the specificity of EV1 Rev binding.

Radiolabelled RNA was obtained by transcription from linearised templates as described above. Varying concentrations of unlabelled UTP were incorporated into each of five separate reactions using the sense template tT7. In addition, the reaction was carried out at 20°C, as lower temperature has been shown to promote the synthesis of full length product (Krieg, 1991). The results of this experiment are depicted in figure 5.5. It is immediately obvious that this template gives rise to a considerably higher yield of product than was previously obtained with pCCR6. The average percentage incorporation of labelled UTP achieved in the absence of unlabelled UTP was 82.4 +/- 5.6% (mean +/- standard deviation for three independent reactions). This figure is within the expected range quoted by the transcription kit manufacturer. The first fifteen nucleotides transcribed by T7 polymerase from the tT7 template are as follows:

GGG CGA AUU GGC CAA

The presence of only two positions requiring the limiting uridine nucleotide may explain the high rate of incorporation achieved by this vector, in contrast to transcription from pTZ18R. Moreover, even with no unlabelled UTP, the proportion of truncated transcripts obtained is low, with a major contaminating species of approximately 300 bases. This species is virtually eliminated in the presence of 25μM cold UTP. Although the presence of this volume of unlabelled nucleotide reduced the percentage incorporation of the label to 25.8% (figure 5.5a), it was found that increasing the reaction time to two hours, and adding a second aliquot of enzyme after one hour increased the average incorporation to 65.8 +/- 3.6%. The

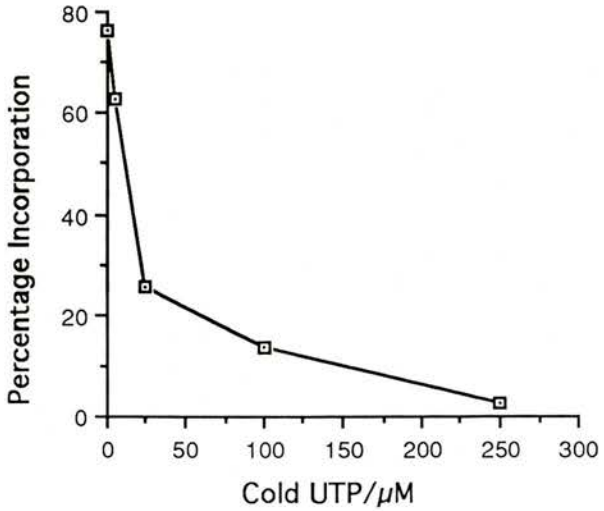
Figure 5.5: Transcription from the pGEM11/RRE Vector

RNA was synthesised from the tT7 template in the presence of a range of concentrations of unlabelled UTP, as described in the text. Reaction products were analysed for the efficiency of label incorporation and for the presence of full length transcripts.

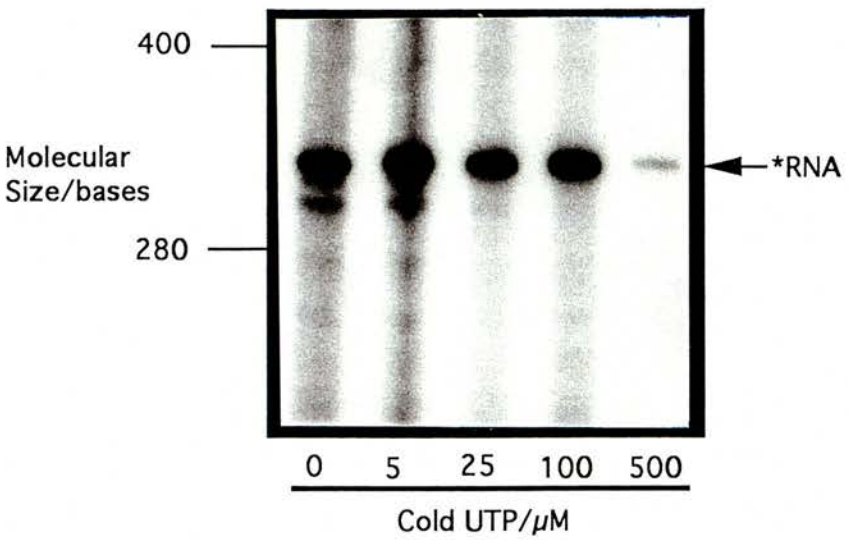
A The efficiency of reactions was analysed by TCA precipitation of synthesised RNA, and scintillation counting of the amount of incorporated label. The percentage of incorporated label is plotted against the concentration of unlabelled UTP used in the transcription reaction.

B Reaction products (~5000cpm) were analysed by electrophoresis through a 6% denaturing polyacrylamide gel. The result was visualised by autoradiography. Labelled transcripts migrating with the expected full length size (329nt) are denoted by an arrow. An insufficient volume of product from the 500μM reaction was loaded on this gel.

A


Percentage Incorporation
80
60
40
20
0
0
50
100
150
200
250
300
Cold UTP/μM

B


400
Molecular
Size/bases
280
*RNA
0
5
25
100
500
Cold UTP/μM

importance of the purity of the transcript for use in functional assays meant that it was decided to continue with transcription reactions containing 25μM cold UTP (figure 5.5b). The molarity of labelled UTP present in the reaction was 6.25μM; the molar ratio of unlabelled to labelled nucleotide in the reaction was therefore 4:1. Assuming that there was no bias in incorporation, each transcript would contain 20% radiolabelled uridine nucleotides. Typically, 250-300ng of RNA were produced per reaction, with the specific activity of labelled transcript in the range 2.5-3 x 10^7 cpm/μg (counting efficiency assumed to be 100%, hence dpm are equivalent to cpm). Antisense RNA, produced from the tSP6 template, was similarly pure, and contained an average 65.5 +/- 4.6% incorporated label (two independent reactions). A template included with the transcription kit was utilised as a second control source. This plasmid was based on the pTRIPLEscript vector, containing a 250bp insert derived from the murine β actin gene. Transcription from this vector with T3 polymerase generates a 325b RNA product. This is almost identical in size to the sense and antisense RRE RNA, and was used as an internal marker control for gel electrophoretic examination of the products of transcription.

The DNA template was digested by incubation with RNase free DNase to terminate transcription, and unincorporated nucleotides were separated from the probes by two rounds of phenol (pH5) extraction and ethanol precipitation. This procedure resulted in the percentage of incorporated label rising to over 80%.

5.4 Rev Binding Assays

5.4.1 Conditions for Binding

The basis of RNA binding assays for analysis of EV1 Rev function was the creation of conditions to allow an equilibrium of protein:RNA interactions to occur, with subsequent examination of the equilibrium by detection of complexed RNA by its reduced mobility during gel electrophoresis, or by its retention on nitrocellulose filters. The reaction conditions chosen were based on the results of published analysis of the *in vitro* RNA binding activity of HIV-1 Rev (Heaphy *et al.*, 1990; Holland *et al.*, 1990; Cook *et al.*, 1991). Partially purified GSTRev was incubated at a range of concentrations (25nM-1.275μM) in binding buffer in moderate ionic conditions (20mM Tris.HCl pH8; 50mM KCl) with yeast tRNA (2μg) to reduce non-specific binding. Labelled RNA probe (~20,000cpm, typically 7.5fmol of 320-330nt

transcript) was subsequently added. The binding reaction was set up in a small volume (20µl) to encourage the formation of equilibrium conditions, and incubated at 25°C for ten minutes prior to analysis.

5.4.2 Gel Mobility Retardation Assays

Analysis of protein:RNA complex formation by the direct electrophoretic separation of complexed and free labelled RNA on non-denaturing, low percentage polyacrylamide gels was developed by Konarska and Sharp (1986) for the study of spliceosome formation. These experiments involved the use of whole cytoplasmic extracts as the protein component. Modifications to the original procedures have been made for the identification of complexes formed by purified protein preparations, including the influenza A virus NS1 protein (Qiu and Krug, 1994), HTLV-1 Rex (Bogerd *et al.*, 1991) and HIV-1 Tat (Dingwall *et al.*, 1990) and Rev (Daly *et al.*, 1989; Cochrane *et al.*, 1990a; Daefler *et al.*, 1990; Holland *et al.*, 1990; Malim *et al.*, 1990; Cook *et al.*, 1991; Kjems *et al.*, 1991a).

GSTRev/RNA binding reactions were diluted in running buffer, containing 15% glycerol for stability, and electrophoresed through 6% native polyacrylamide gels. The results of binding of GSTRev to sense RRE RNA and the β-actin control RNA are illustrated by figure 5.6a,b. A retarded complex appears to be formed by sense RRE RNA in the presence of 210ng GSTRev (~220nM). The mobility of this complex is further reduced by the presence of higher concentrations of GSTRev. A different pattern is apparent with control RNA. Although the mobility of the major portion of RNA is unaffected by the concentration of GSTRev, a small retarded signal is apparent at all protein concentrations (arrow). The uniformity of the retarded signal with respect to protein concentration, and the degree of retardation, suggests that this is may be a non-specific phenomenon. This experiment therefore provides preliminary evidence for specific interactions between GSTRev and RNA corresponding to the predicted RRE. However, the degree of degradation of RNA probe observed on binding and electrophoresis makes interpretation difficult. It is clear from the sense RRE reaction that intermediates of degradation are present regardless of the presence of protein. To determine whether RNases were active during the binding/incubation and/or electrophoresis phase of this assay, a time course of degradation was performed by incubating aliquots (10000cpm) of sense RRE probe with ~630ng of GSTRev and boiling the reaction to terminate possible

Figure 5.6: Analysis of GSTRev Function by Gel Mobility Retardation

Approximately 20,000 TCA-precipitable cpm of ^{32}P labelled RNA probe (~7.5fmol/0.37nM) was incubated in binding buffer (20mM Tris.HCl, pH 7.5. 50mM KCl, 1mM DTT, 2mM $MgCl_2$, 2μg yeast tRNA, 15U RNasin) with a variable volume of eluted, partially purified GSTRev fusion protein in a total volume of 20μl. After ten minutes incubation at 25°C complex formation was analysed by addition of loading buffer and electrophoresis of a 10μl aliquot through a native, 4% acrylamide gel. The result was visualised by autoradiography.

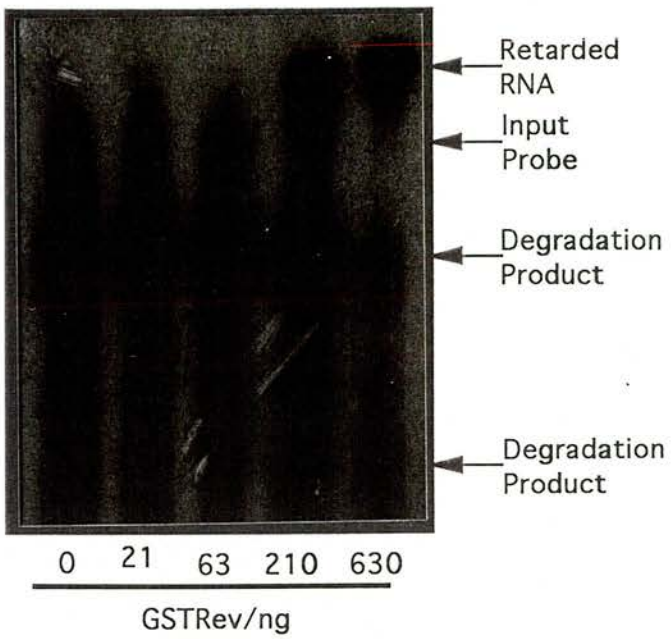
A Sense RNA probe

B Control (β-actin) RNA probe

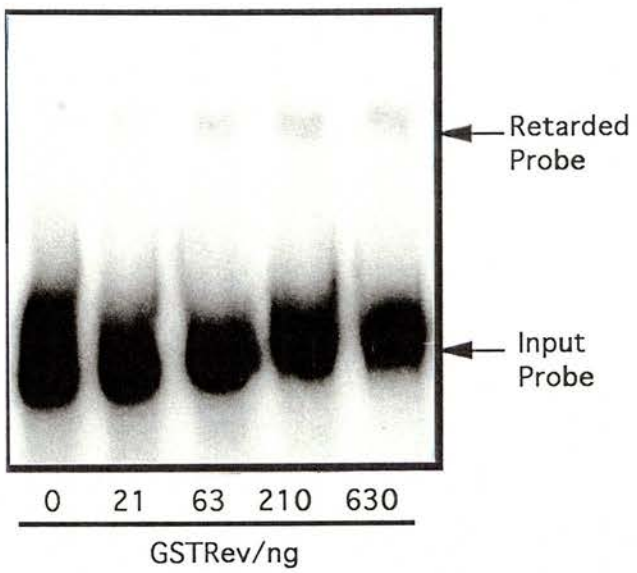
The input probe and retarded signals are indicated, as are degradation products due to RNase activity.

C Analysis of the effect of the addition of the GSTRev fusion protein to RNA stability. Sense RNA (10,000 cpm) was incubated at 25°C in binding buffer containing ~630ng of GSTRev (total ~3.2mg protein), for 5-60 minutes. Reactions were terminated by the addition of denaturing loading buffer (includes formamide) and boiling. Electrophoresis through a 6% denaturing polyacrylamide gel was followed by autoradiography. The full length sense probe is indicated.

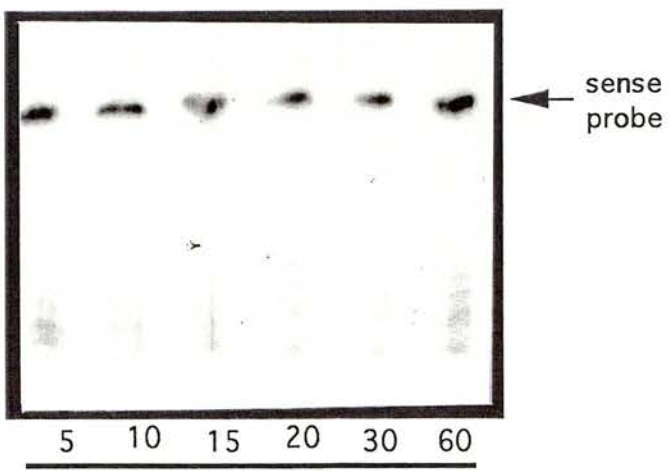
A


Retarded
RNA
Input
Probe
Degradation
Product
Degradation
Product
0 21 63 210 630
GSTRev/ng

B


Retarded
Probe
Input
Probe
0 21 63 210 630
GSTRev/ng

C


sense
probe
5 10 15 20 30 60
INCUBATION TIME/minutes

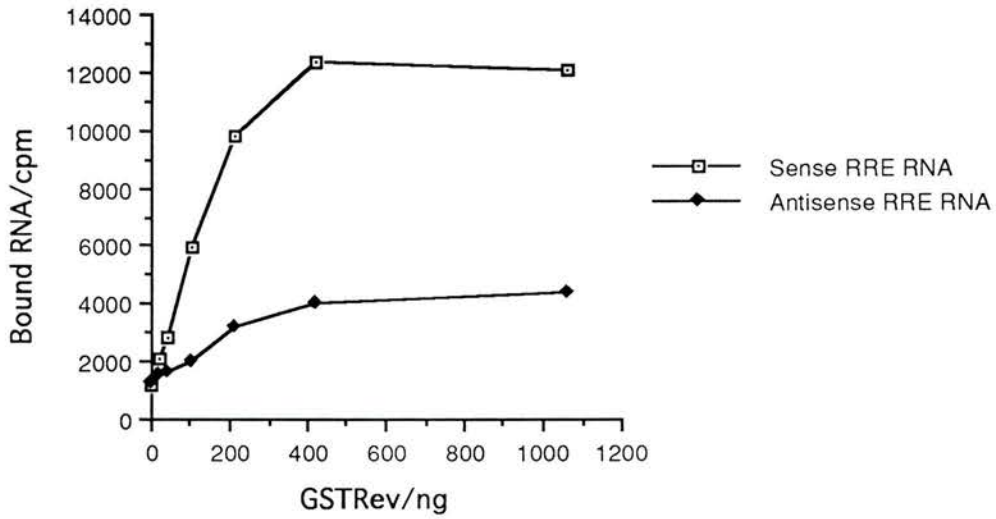
degradation. The results were visualised by electrophoresis through a denaturing (8M urea) 6% polyacrylamide gel, with subsequent autoradiography, and quantified by TCA precipitation of incorporated label. It was assumed that denaturing conditions would inhibit further RNase activity. From figure 5.6c it can be seen that little degradation occurred. The level of incorporated label was found not to fall significantly. Thus, it would appear that native gel conditions may have been responsible for probe degradation. Although strict precautions were taken against RNase contamination, ie all buffers were made up with diethylpyrocarbonate (DEPC) treated water, and glassware treated similarly, the large buffer volumes and the degree of manipulation and time involved in PAGE analysis meant that complete inhibition of RNase activity was difficult. Experiments with antisense RRE RNA were unsuccessful, due to the almost complete degradation of the probe during electrophoresis. The presence of degradation products meant that quantification of the results of retardation assays by densitometric analysis was not feasible. Moreover, this assay proved to be poorly reproducible. Ribonucleoprotein complex dissociation may have been responsible for this, despite the use of the binding buffer as electrophoresis buffer. Unavoidable delays in loading reactions onto gels, and subtle differences in local buffer conditions might have resulted in differences in the stability of complexes, leading to dissociation prior to, or during electrophoresis. Attempts to increase stability by the addition of glycerol to 4% v/v within the gel (Nalin *et al.*, 1990; Bogerd *et al.*, 1991) were unsuccessful. Given the problems associated with the gel mobility retardation assay, it was decided that the filter binding assay would be employed as an alternative.

5.4.3.1 Nitrocellulose Filter Binding Assays

Filtration of small radiolabelled RNA transcripts through a 0.45μM nitrocellulose membrane will result in significant retention on the filter only if the RNA is complexed with protein. Scintillation counting of the retained probe allows complex formation between protein and RNA to be quantified. Filter binding assays were developed in an attempt to identify the DNA binding specificity of the *E. coli* lac repressor (Riggs *et al.*, 1968). Subsequently, this method was adapted for use with RNA systems by Carey and co-workers (1983), during investigation of the characteristics of binding between the bacteriophage R17 coat protein and the RNA genome. Filter binding has the advantages of ease and rapidity,

Figure 5.7: Preliminary Nitrocellulose Filter Binding Assay

Variable amounts of GSTRev were incubated with 20,000 cpm of two RNA probes, sense and antisense RRE RNA (from pGEM11/RRE) as described in figure 5.6. The whole reaction volume was filtered through pre-wetted 0.45μM nitrocellulose filter using a Schleicher and Schuell 'slot blot' apparatus. Filters were washed twice with binding buffer (20mM Tris.HCl pH 7.5, 50mM KCl) and air-dried. Trapped ribonucleoprotein complexes were quantified by scintillation counting of individual sections of filter. The results are plotted as the amount of retained radioactivity (cpm) against the mass of GSTRev in each reaction.


14000
12000
10000
8000
6000
4000
2000
0
Bound RNA/cpm
0
200
400
600
800
1000
1200
GSTRev/ng
Sense RRE RNA
Antisense RRE RNA

which should reduce contaminating RNase activity. Furthermore, a number of reactions can be carried out simultaneously, which should reduce inter-experimental variation.

Preliminary experiments to determine the validity of this assay were performed. Varying concentrations of GSTRev were incubated with labelled sense and antisense RRE RNA (~20,000cpm) obtained from pGEM11/RRE, in the presence of nonspecific competitor RNA (yeast tRNA, 2µg/20µl reaction). The results are illustrated in crude form in figure 5.7. A proportion of the probe is clearly retained even in the absence of protein. The sense RRE probe is retained on the filter at low GSTRev concentrations. This is suggestive of specific ribonucleoprotein complex formation. In contrast, the antisense probe requires higher concentrations for significant retention. Retention of the antisense probe does not reach a plateau, suggestive of non-specific interactions. Further experiments were carried out in order to define assay parameters.

5.4.3.2 Assay Parameters

The accuracy and hence the utility of the filter binding assay depends on the amount of retained label accurately reflecting the equilibrium binding between protein and RNA achieved prior to filtration. The mechanics of the assay may distort this equilibrium in two main ways. Firstly, leaching of RNA from complexes retained on the nitrocellulose membrane would lead to an underestimation of binding. The number of washes after filtration might be expected to affect leaching. Moreover, if the complex half life did not greatly exceed the time taken for filtration, then dissociation would occur. Secondly, inappropriate, non-specific binding of RNA by membrane bound protein would lead to an overestimation of binding. If a sufficiently large protein concentration was added, blocking of the filter might result in large scale retention of labelled probe. The relative impurity of the GSTRev preparation meant that the total mass of protein added was high: hence this latter point might be significant in the current context.

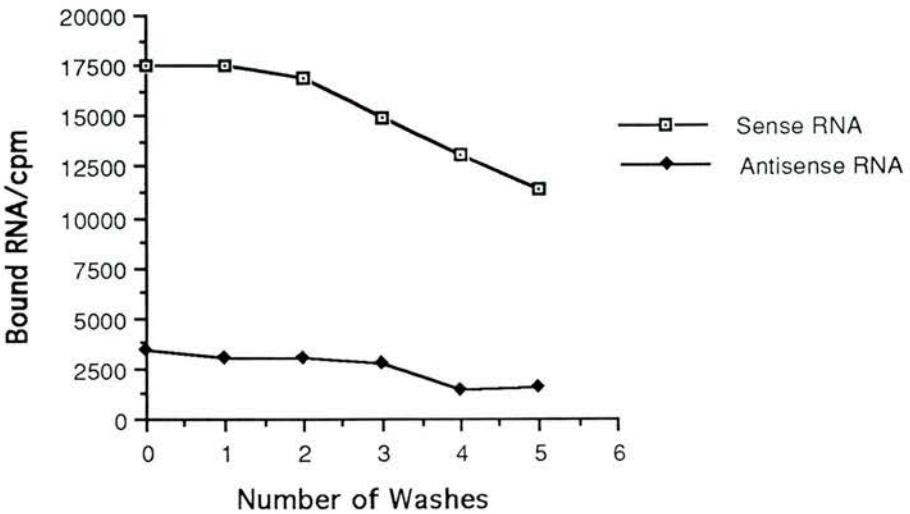
The parameters for the reliability of the assay were therefore investigated. To test for leaching of bound probe, an experiment was carried out using standard procedures, in GSTRev excess (500nM), but varying the number of post-filtration washes from none to five. The time spent on the membrane was directly proportional to the number of washes, so that complex dissociation due to all factors could be studied. The results are shown in figure 5.8a.

Figure 5.8: Filter Binding Assay Parameters

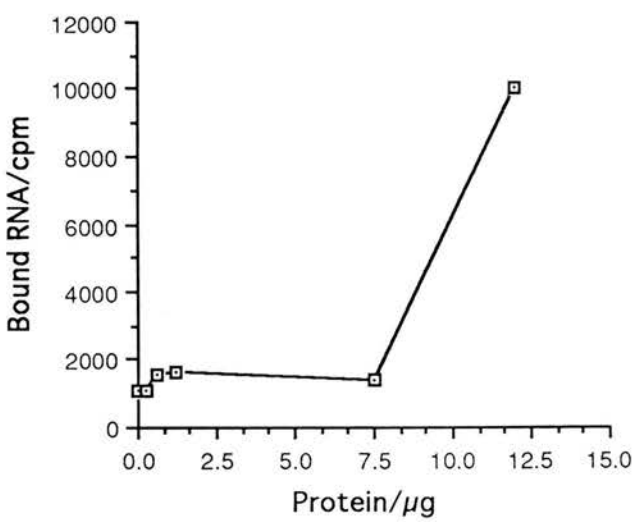
A Standard filter binding assay, using 500nM GSTRev and 20,000 precipitable cpm sense and antisense RNA probes (~8fmol sense/7.5fmol antisense). The number of post-filtration washes was varied from none to five, and the retained RNA analysed by scintillation counting. The results are plotted as retained RNA (cpm) against the number of wash steps.

B Standard binding reactions with variable protein concentrations but without labelled RNA were set up, and filtered. Subsequently, 20,000 cpm of sense RNA in a total volume of 20µl was added to each well, and filtration repeated. Bound RNA was counted after a single wash step. Results are plotted as the level of retained RNA (cpm) against the total mass of input protein.

A


20000
17500
15000
12500
10000
7500
5000
2500
0
Bound RNA/cpm
0
1
2
3
4
5
6
Number of Washes
Sense RNA
Antisense RNA

B


12000
10000
8000
6000
4000
2000
0
Bound RNA/cpm
0.0
2.5
5.0
7.5
10.0
12.5
15.0
Protein/μg

The first two washes appear to have no effect on the level of bound RNA. Subsequently, there is a shallow decline in binding, which is, perhaps surprisingly, more evident with sense RRE RNA than with the antisense RRE RNA. It is impossible to determine whether the reduced binding is due to leaching caused by repeated washing and filtration, or a consequence of dissociation kinetics. However, this experiment suggests that loss of bound RNA is not significant up to two washes. Thus, two wash steps were retained within the experimental procedure.

To test for the effect of pre-bound protein on filtration of free probe, binding reactions with varying protein concentrations were set up and filtered without the addition of labelled RNA. An aliquot of labelled, sense RRE RNA (~20,000cpm) was then added to wash buffer within each chamber, and filtration repeated. The amount of bound RNA was counted, and is illustrated by figure 5.8b. It is clear that the presence of <8μg total protein does not reduce the filtration of RNA under these conditions. However, pre-binding of 12μg of protein resulted in ~50% retention. This suggests that blocking of the filter can occur at very high protein concentrations. These concentrations were avoided during further analysis of GSTRev binding.

5.4.3.3 GSTRev RNA Binding Affinity

A series of filter binding experiments were carried out in order to investigate the binding of GSTRev to RNA targets. Three different RNA probes were used; the sense and antisense RRE probes derived from tT7 and tSP6 respectively, and the β-actin probe derived from the control template. Initial experiments were performed in the presence of 2μg/20μl reaction of yeast tRNA. For data analysis, the concentration of GSTRev was determined by Biorad assay, and the percentage of full-length product by densitometric analysis. The molarity of GSTRev was thence calculated on the basis of a predicted molecular weight of 47kDa. Typically, 1μl of GSTRev preparation contained 1.2mg/ml protein, of which 20% was full length fusion protein (240ng). Addition of 1μl to a 20μl binding reaction resulted in a final GSTRev molarity of approximately 250nM. A standard probe input of ~20000cpm was maintained. The specific activity of each probe was determined, and the mass of input RNA calculated. The results of these studies are depicted by figure 5.9. Figure 5.9a demonstrates the affinity of GSTRev for each labelled probe. This graph was computed on the basis of an

Figure 5.9: The Affinity of GSTRev for Radiolabelled RNA Probes

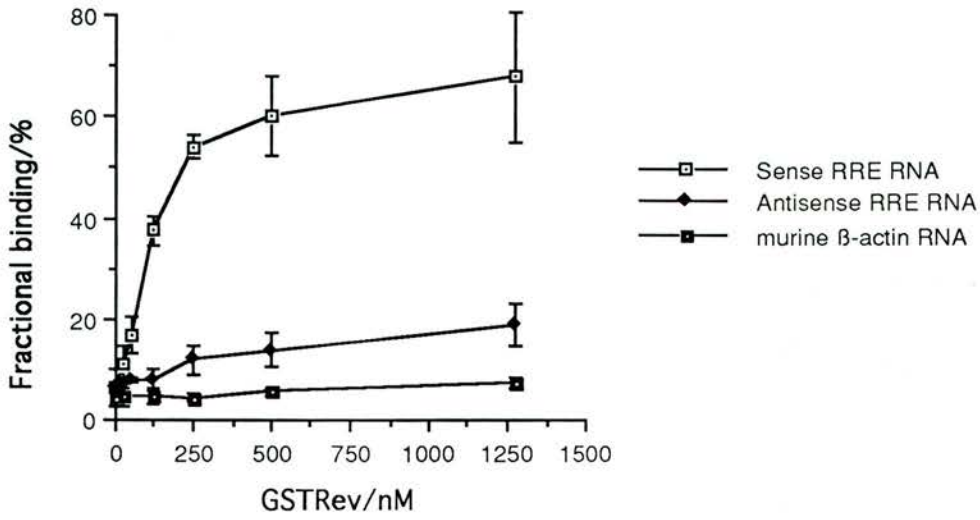
Standard binding reactions were set up, with varying concentrations of GSTRev (25-1275nM). Three input labelled RNAs were used, the sense and antisense RRE RNAs and the β-actin RNA. RNA input was standardised to 7.5fmol (18000-20000cpm).

A Results are plotted as the percentage of total input RNA retained at each concentration (fractional binding) against the GSTRev concentration. Figures represent the mean +/- standard deviation from three independent reactions.

B Variation in results obtained from the filter binding assay. Three separate reactions were performed at each protein concentration using the same substrate sources, to represent the intra-assay variation. The results presented in A are used to represent inter-assay variation. Figures refer to the mean +/- standard deviation.

C Standard binding assay, using non-fusion GST as the protein substrate. Results are derived from a single experiment.

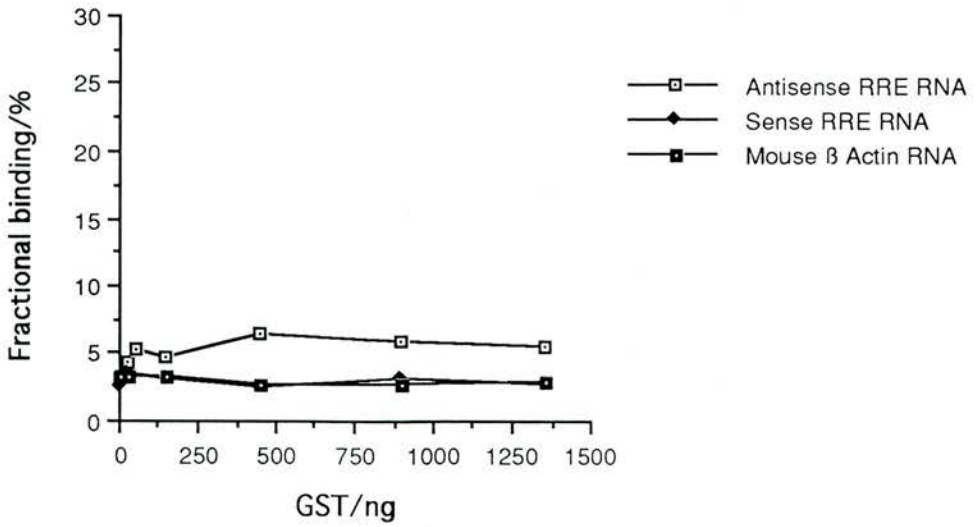
A



B

GSTRev /nM	INTRAASSAY VARIATION				INTERASSAY VARIATION			
	SENSE		ANTISENSE		SENSE		ANTISENSE	
0	5.3	0.11	5.5	0.25	6.5	0.42	6.3	0.4
25	8.1	0.15	6.2	0.1	11.2	3.2	6.8	0.5
50	14.5	0.3	8.6	0.26	16.8	3.6	7.9	0.65
125	33.6	1.26	8.9	0.3	37.4	2.7	7.8	2.2
250	52.8	1.15	12.1	0.62	54.1	2.2	11.8	2.7
500	61.2	0.9	14.2	0.23	60.3	7.8	13.8	3.5
1275	65.3	0.9	21.5	1.37	68.1	13	18.7	4

C



average of three independent functional assays, involving distinct protein and RNA probe preparations. The mean and standard deviation of these results are shown. The results are plotted in the form of fractional binding, that is the percentage of input probe bound at each distinct protein concentration. GSTRev has a relatively high affinity for the sense RNA (S-RRE) probe. Probe retention occurs on input of low protein concentrations, and rises rapidly. A plateau is reached at approximately 500nM GSTRev. A proportion of the S-RRE probe remains unbound, even in the presence of saturating GSTRev concentrations. This proportion is variable between different preparations. GSTRev also demonstrates binding to the antisense probe, A-RRE. However,binding characteristics are different, with binding slowly increasing with protein concentration. Only ~20% of this probe is bound at a GSTRev concentration of 1.25μM. GSTRev effectively fails to bind to the control probe, β-actin RNA.

Variation between experiments was examined to investigate the reproducibility of this assay. Figure 5.9b tabulates the mean and standard deviation of three data sets derived from independent reactions (inter-assay variation) or three different assays from the same reaction (intra-assay variation). Variation is clearly significant between different reactions, but appears to be minimal with respect to assays performed using the same reactions. The assay itself is therefore reproducible. Systematic errors introduced by the requirement for estimation of both the protein concentration and purity, and in the addition of probe may in part explain such inter-assay variation.

To investigate which component of the GSTRev fusion chimaera was responsible for RNA binding, a separate experiment was performed using purified GST as the input protein. GST fails to bind to the sense RRE or β-actin control probes, although the antisense probe was retained to a slight degree (figure 5.9c). As only a single experiment was performed, the relevance of this was difficult to gauge.

The background level of probe retention was low throughout these assays. The average retention in the absence of protein was 4 +/- 1.6% for β-actin RNA; 6.5 +/- 0.4% for S-RRE, and 6.3 +/- 0.4% for A-RRE (from three independent reactions). To calculate the degree of affinity for RNA, background levels were subtracted from the totals. The dissociation constant (Kd) is a measure of affinity, and is here defined as the protein concentration at half maximal binding. This figure is only valid if the concentration of one binding substrate is in excess over that of the other. For the sense RRE probe, the saturation level was 60% of input probe. Hence, the Kd for GSTRev binding to S-RRE under these conditions was estimated at

120nM. At this concentration, GSTRev is present in an approximate 325 molar excess over the sense RNA probe (120nM compared to 370pM).

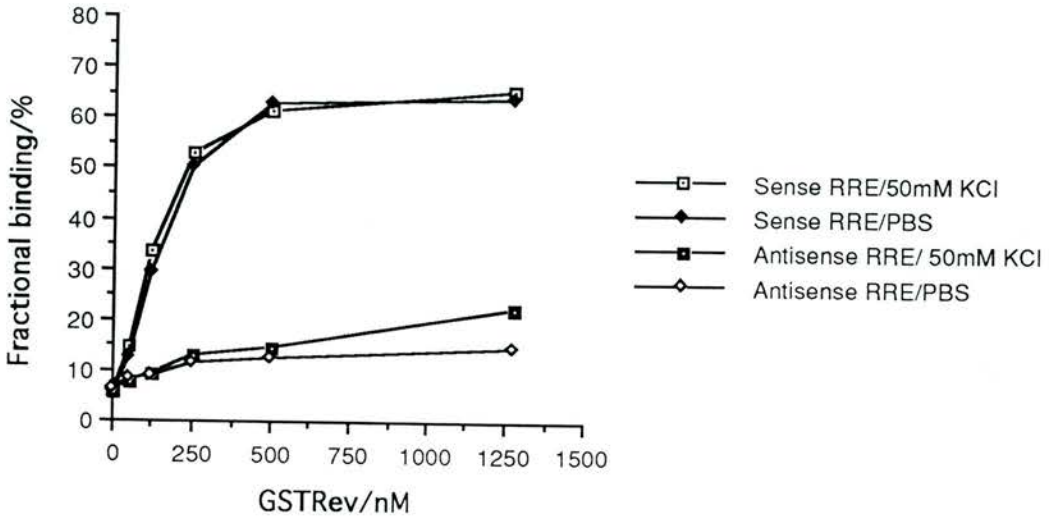
5.4.3.4 GSTRev RNA Binding Specificity

To investigate the specificity of GSTRev RNA binding, a series of preliminary experiments were performed in which components of the binding reaction were varied. The arginine-rich putative basic domain of EV1 Rev might be expected to mediate non-specific, electrostatic interactions with RNA. This is the likely basis for affinity to the antisense RRE probe. Altering the ionic conditions within the binding buffer would affect such non-specific interactions, but have little influence on specific binding. To determine the effect of ionic strength on binding by GSTRev, binding was carried out in 50mM potassium chloride as standard, and in phosphate buffered saline (PBS), which contains 137mM sodium chloride, and 26.8mM potassium chloride. The result is presented in figure 5.10. Whilst the change in buffer conditions did not alter the binding curve of GSTRev to sense RRE RNA, there is a slight decrease in binding to the antisense probe in higher ionic conditions, most noticeably at higher protein concentrations. It must be emphasised that, due to pressure of time, only a single experiment was performed. Further investigation would be required to attempt a statistical analysis of this data. Furthermore, a study of binding activity by titration of ionic conditions would be required for full analysis.

The level of unlabelled RNA required to compete out the binding of GSTRev to labelled probe is a measure of the specificity of binding. Reduction in binding at low concentrations of competitor indicates a low specificity binding, whereas highly specific binding requires a high concentration of competitor for inhibition. Competitor RNA was added prior to the addition of probe RNA in all experiments. Two competitor species were available. Yeast tRNA, included in the standard reactions to act as a 'sink' for non-specific interactions, was added at a range of concentrations. The effect of titrating yeast tRNA on binding is illustrated by figure 5.11a. The concentration of protein used in these experiments was constant, and set at 480-500nM. The molarity of yeast tRNA was determined by assuming that the average molecule contains 80 nucleotides. The membrane retention of sense probe is relatively unaffected by the concentration of tRNA, within the range used here. However, binding to antisense RRE is strongly dependent on the level of tRNA competitor. In the

Figure 5.10: The Effect of Buffer Conditions on RNA Binding by GSTRev

Binding reactions between GSTRev and the sense and antisense RNA probes were performed using standard buffer conditions, or by replacing the Tris/KCl with sterile phosphate buffered saline. Results were derived from a single experiment.


Fractional binding/%
80
70
60
50
40
30
20
10
0
0
250
500
750
1000
1250
1500
GSTRev/nM
Sense RRE/50mM KCl
Sense RRE/PBS
Antisense RRE/ 50mM KCl
Antisense RRE/PBS

absence of competitor, a high proportion (~60%) of antisense RNA is retained. Retention is dramatically reduced above 0.4μM tRNA. The concentration of tRNA required for 50% binding inhibition can be estimated from this result to be ~1.05μM; however, more data points would be required for an accurate determination of this value, as there is a steep reduction in binding between 0.36μM and 3.6μM yeast tRNA. The mass of specific probe used in these experiments was ~0.8ng. This equates to a molarity of 0.37nM for each probe (which differ by 7/329 nucleotides: assume average nucleotide mass of 330Da). Hence, 50% reduction in binding to the antisense probe is achieved in the presence of a ~2800 molar excess of yeast tRNA.

A second competitor species was available in the form of unlabelled sense and antisense RNAs. Theoretically, binding to labelled sense probe should be reduced by a simple molar excess of specific unlabelled competitor. It is assumed that the binding protein does not discriminate between labelled and unlabelled forms. In practice, the concentration of protein will affect the level of competition. Unlabelled ('cold') RNA was prepared by increasing the concentration of unlabelled UTP in the transcription reaction to 500μM; thus all four ribonucleotides were present at saturating concentrations. Transcripts were purified by phenol extraction and ethanol precipitation, and the concentration estimated by spectrophotometry. An aliquot of each transcript was visualised by denaturing gel electrophoresis (figure 5.11b). RNA of the expected size was clearly present. Cold sense RRE RNA was used as a competitor in reactions between 480-500nM GSTRev and 0.35-0.4nM labelled probe. Non-specific competitor (yeast tRNA) was excluded from these reactions. The result of this experiment is illustrated by figure 5.11c. Unexpectedly, the fractional binding of GSTRev to both the sense and antisense RRE RNA probes is almost totally unaffected by the titre of cold sense RRE RNA. Although there may be a slight fall in antisense RRE binding, it is clearly not a significant reduction. The highest cold RNA concentration used, 100nM, corresponds to an approximate 250-275 molar excess of cold over 'hot' RNA. An identical experiment with antisense competitor gave similar results (data not shown). Further experiments, to analyse the effect of reducing the protein concentration and of increasing the titre of competitor RNA, were indicated: unfortunately, pressure of time did not allow for completion of this work.

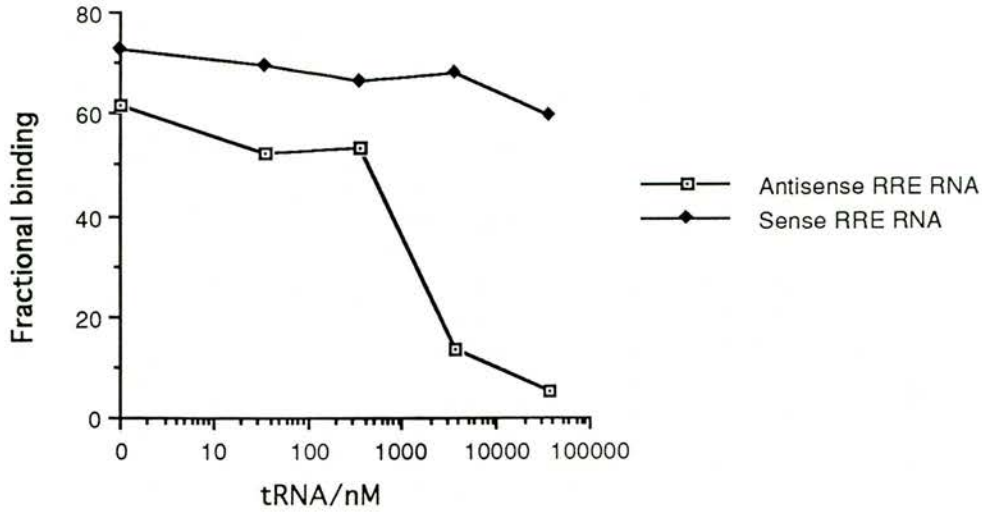
Figure 5.11: The Specificity of GSTRev Binding: Competition Experiments

A The effect of the level of heterologous RNA on GSTRev binding was investigated by standard binding reactions, with the pre-incubation step containing varying concentrations of yeast tRNA (0-36μM - 0-20μg). The concentration of GSTRev was 500nM, and 20,000cpm of labelled probe were subsequently added. Results are plotted as the fractional binding of input labelled probe against the concentration of yeast tRNA.

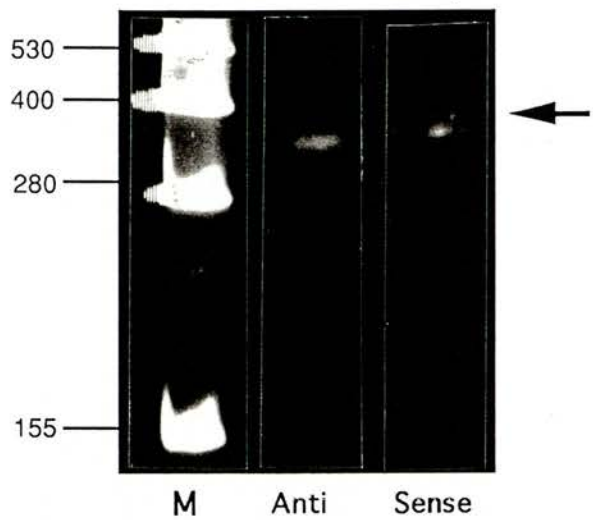
B Transcription of unlabelled ('cold') sense and antisense RRE RNA. Transcription reactions were assembled with all four ribonucleotides present at saturating concentration (500μM). Products of transcription were analysed by electrophoresis through a 8% denaturing acrylamide minigel, with visualisation by staining in ethidium bromide.

C Competition experiment performed as described in A above, except that unlabelled competitor was sense RRE RNA. The fractional binding of labelled probe is plotted against the concentration of unlabelled RNA competitor.

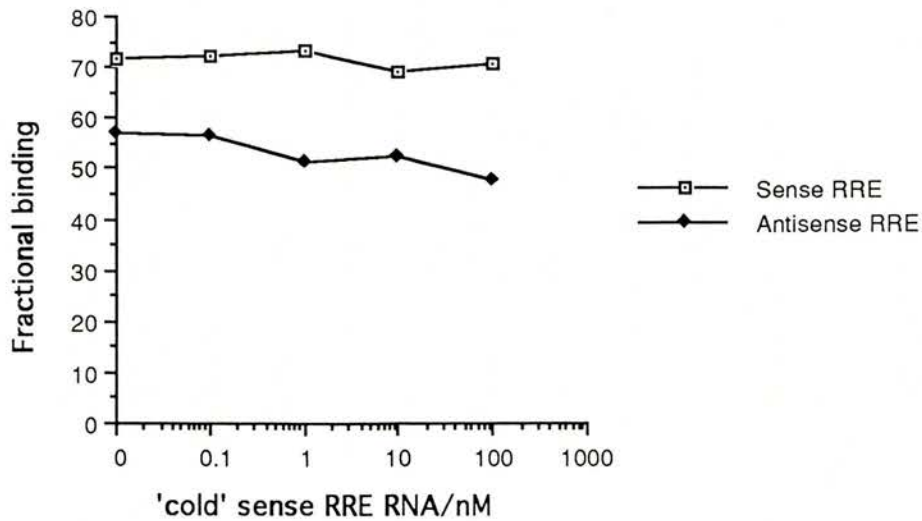
A


80
60
40
20
0
Fractional binding
0
10
100
1000
10000
100000
tRNA/nM
Antisense RRE RNA
Sense RRE RNA

B


530
400
280
155
M
Anti
Sense

C


80
70
60
50
40
30
20
10
0
Fractional binding
0
0.1
1
10
100
1000
'cold' sense RRE RNA/nM
Sense RRE
Antisense RRE

5.5 Discussion

This chapter has reported the generation of RNA probes for the attempted characterisation of the EV1 Rev responsive element. Binding of GSTRev fusion protein to these probes was analysed by assaying for the presence of ribonucleoprotein complexes. Although a gel mobility shift assay proved technically unworkable, results were generated by the use of a nitrocellulose filter binding assay. Evidence was obtained to suggest that data derived from this assay accurately reflected the degree of solution binding of protein and RNA. Conclusions drawn from the results presented in this chapter are subject to several caveats, and further study to confirm the specificity of the Rev/RRE reaction in the absence of contaminating proteins and RNA substructures will be required.

5.5.1 GSTRev Binding Affinity

Saturation binding experiments were performed in order to investigate the affinity of GSTRev for RNA probes derived from the EV1 viral genome. Addition of GSTRev resulted in membrane retention of the RNA probe containing the putative RRE in the sense orientation during filtration. The estimated dissociation constant (Kd), a measure of binding affinity, was 120nM. This figure, and other conclusions drawn from this work, must serve as a preliminary estimation of binding affinity, given the following important points concerning experimental design and conditions. The protein substrate was only ~20% pure, and contained truncated fusion fragments which may be capable of binding; this would lead to an underestimate of the concentration of functionally competent protein molecules within the reaction. The RNA probe contained extraneous sequences, including an additional stem-loop structure which may have interfered, positively or negatively, with binding. The Kd should be largely unaffected by the concentration of input probe: however, these experiments were performed at a single input probe concentration. At the Kd point GSTRev was present in an approximate 325 molar excess over the sense RRE probe (GSTRev 120nm: RNA ~0.37nM). The effect of altering this concentration on the Kd should be examined to validate the estimated figure. To eliminate the possibility that differential degradation of probes occurs during filtration, such that the antisense RNA has lower stability, the filtration run-through could be collected and analysed for RNA stability be gel electrophoresis. To confirm the protein specificity of the

binding reaction, an anti-Rev antiserum could be used to block binding: the anti-peptide antiserum described in chapter four has specificity for the carboxy-terminal protein region and would not be suitable for this purpose.

GSTRev was also able to mediate partial retention of antisense RRE RNA at high concentrations. In the absence of non-specific competitor, high levels of antisense probe were retained at saturating GSTRev concentrations. This RNA is predicted to form extensive secondary structure. The failure to similarly interact with the mouse β-actin probe suggests that GSTRev has a general affinity for regions of secondary structure. The superficial similarity between the antisense and sense probes with respect to the presence and organisation of stem-loop structures suggests that the primary Rev recognition element is dependent on primary structure or on distinct substructures not shared by the two probes. A low level of transcription may occur from the incorrect strand during RNA synthesis (Schenborn and Meirendorf, 1985): thus there may be contamination of the antisense RNA preparation with sense structures, which may mediate the observed low level binding. If a template error level of 0.2% is assumed for the polymerases, then the concentration of heterologous probe would be in the region of 0.75pM per binding aliquot. Reactions using this concentration of specific sense probe might indicate whether contamination of the antisense preparation is sufficient to mediate the partial binding observed, or whether GSTRev possesses affinity for the antisense probe in its own right.

Thus, given the structural and genomic locational similarity of the EV1 RNA element to the HIV/SIV RREs, and the high affinity of GSTRev for a probe containing this element, it can be tentatively concluded that the EV1 *env* sequences 7935-8216 contain a GSTRev binding element effective only in the sense orientation. Further delineation of this element, and in particular the definition of possible binding sites of different affinities, would require analysis using truncated RNA fragments. The pGEM11/RRE construct might be suitable for such a study, by utilising unique restriction endonuclease sites within the EV1 insert to linearise the plasmid as a transcriptional template. Excision of the extraneous, vector-derived sequences from the RNA probe would remove the uncertainty surrounding the precise role of the virus-derived sequences in protein binding. Incorporation of a polymerase promoter within a primer used to amplify the putative RRE by PCR would result in the production of RNA containing viral sequence only. This 'promoter tagging' technique has been utilised by other authors working on the HIV-1 Rev/RRE interaction (Holland *et al.*, 1990; Cook *et al.*, 1991). It

should be noted that the relatively large region of extraneous sequence derived from the pGEM vector may have improved transcription efficiency compared to the pTZ18R vector, by providing an initiating sequence with few uridine nucleotides. Alternatively, synthetic oligonucleotides could be employed as the RNA substrate. For this approach to be technically feasible, further localisation of a EV1 Rev high affinity binding element would be required. Synthetic peptides corresponding to the HIV-1 Rev arginine-rich domain are able to bind the RRE with similar affinity and specificity as the intact molecules (Kjems *et al.*, 1992). Peptides corresponding to the EV1 Rev basic domain, and several variants, were designed for a similar study. However, technical problems, due to the high arginine content, were encountered by several commercial chemical companies in the manufacture of these peptides.

The estimated Kd for HIV-1 Rev binding is 0.8-5nM (Heaphy *et al.*, 1990; Holland *et al.*, 1990; Cook *et al.*, 1991). A high Kd may be necessary for functional discrimination of viral, target RNA from an excess of competing cellular RNA within the infected cell nucleus. However, specific discrimination of target from background molecules can be achieved with a relatively low Kd: the ribosomal S4 protein has a Kd of 14mM for 16S ribosomal RNA, its molecular target (Vartikar and Draper, 1989). The estimated binding constant for EV1 GSTRev of 120nM, is in the region of two orders of magnitude lower than that of HIV-1 Rev. Although this might suggest a reduced affinity for target RNA exhibited by the EV1 molecule, the aforementioned points concerning the validity of this data mean that over interpretation should be avoided. Possible modulation of binding affinity by the GST fusion partner could explain the lower binding constant reported. Cullen and co-workers found that 27ng of free HIV-1 Rev protein were functionally equivalent to 330ng of GSTRev (~130ng Rev) in a RNA binding gel retardation assay (Malim and Cullen, 1991). Thus the efficiency of binding of fused Rev was in the order of one fifth of that obtained with the free protein. The validity of GST fusion protein functional studies is discussed in more detail in chapter seven. A source of pure, free EV1 Rev protein would be required to determine how the relevance of the fusion protein data.

5.5.2 GSTRev Binding Specificity

Competition experiments were performed in order to investigate the specificity of

GSTRev binding to RNA ligands. The concentration of competitor ligand required for a 50% reduction in binding is a standard measure of binding specificity. A non-specific competitor, yeast transfer RNA, was shown to inhibit the binding of GSTRev to the antisense RRE ligand. A concentration of ~1.05μM tRNA was required to reduce the fractional binding of antisense RNA by one half. This value is within the range reported for a similar inhibition of HIV-1 anti-RRE Rev binding (0.7-20μM) (Daly *et al.*, 1989; Cook *et al.*, 1991). These figures are not directly comparable, however, as the configuration of the antisense molecules will differ, and mediate different binding affinities. Binding to sense RRE RNA was not significantly reduced in the presence of 36μM tRNA, an approximate 28,000 fold molar excess. Cook and colleagues have reported that a 10^5 fold excess of tRNA is required for a 50% reduction in the binding of HIV-1 Rev to sense RRE RNA (1991). Altering the ionic strength of the binding buffer was performed to further investigate binding specificity. Farrington and colleagues (1991) have shown that the HIV-1 Rev protein can bind both sense and antisense RRE RNA with similar affinities in low ionic strength buffers, but that raising the ionic strength results in a progressive loss of binding to the non-specific probe. Although the different conditions used in this report did not result in significantly altered binding, the difference in ionic strength was relatively minor. The use of greater variations in buffer strength is indicated.

The failure to achieve an inhibition of the binding of GSTRev to labelled probe in the presence of an excess of unlabelled specific competitor was an unexpected finding. However, the use of a saturating protein excess in these experiments probably provides an explanation. If both specific and non-specific RNA binding sites are present within the protein molecules, then the total number of binding sites present in saturating concentrations of protein will be very high. Binding of specific competitor to the high affinity sites of a proportion of these molecules will not disrupt the subsequent binding of either specific or non-specific labelled RNA. Repetition of these experiments with reduction the protein input concentration to the estimated Kd value is indicated.

5.5.3 Fractional Binding

Although a high percentage, 60-70%, of the input sense RRE probe was retained on the filter at saturating GSTRev concentrations, the fractional saturation did not reach 100%. This phenomenon has been widely reported, both during Rev/RRE investigation (Heaphy *et*

al., 1990; Holland *et al.*, 1990), and in other systems (Carey *et al.*, 1983; Qiu and Krug, 1994). Furthermore, the plateau level of fractional binding was found to be the most variable figure within the filter binding assay data set. This latter point suggests that a property of the reaction substrates may affect fractional saturation. A proportion of the protein or RNA input molecules may therefore have not been competent for binding, due perhaps to incorrect folding leading to masking or inactivity of functional domains. Variation in this proportion between preparations would account for the observed variation. It should be noted that to estimate the binding Kd, it was assumed that all molecules within the reaction were competent for binding. A second explanation is that the kinetics of the binding reaction prevent saturation of the RNA. The relative half lives for complex assembly and disassembly would determine the proportion of ligand bound at equilibrium; this will not approach 100% unless the half life for binding greatly exceeds that for disassembly.

5.5.4 The Gel Retardation Assay

Gel mobility retardation assays have been widely employed in the study of HIV-1 Rev. These assays have the benefit of visualisation of the stoichiometry of the binding reaction, as multiple retarded complexes have been observed at varying protein concentrations (Iwai *et al.*, 1992; Daly *et al.*, 1993a). The difficulties here encountered in high resolution of stable ribonucleoprotein complexes by electrophoresis meant that the binding stoichiometry could not be so examined. Reducing the level of gel cross-linking by lowering the bis-acrylamide to acrylamide ratio from 40:1 to 80:1 has been associated with high resolution of complexed RNA (Konarska and Sharp, 1986). The apparent RNase susceptibility of probe under native gel conditions may be countered by more extensive DEPC treatment of glassware and buffers, although the precautions used in this project were already stringent. Attempts to increase complex stability by altering gel conditions, and by running gels at 4°C, were unsuccessful.

5.6 Summary

A 176nt RNA element from within the EV1 *env* gene, immediately downstream of the SU/TM boundary, is predicted to form a stable secondary structure with superficial

resemblance to the HIV/SIV RRE. Generation of this RNA sequence by run-off transcription was found to be dependent on vector context. Highly pure RNA was obtained by transcription from a pGEM11Zf(+) vector containing a PCR derived *env* gene insert. Binding between radiolabelled RNA and partially purified GSTRev fusion protein was monitored by assays designed to detect ribonucleoprotein complexes. Problems with RNase degradation and complex stability negated the use of a gel mobility retardation assay, although preliminary experiments were suggestive of GSTRev binding to a probe corresponding to the putative RRE element. Nitrocellulose filter binding assays were more successful. GSTRev was found to bind with high affinity to sense RRE RNA, and with less affinity to antisense RRE RNA. The dissociation constant for GSTRev binding to sense RNA was estimated to be 120nM. Binding to antisense RNA was dependent on the level of a non-specific RNA competitor, and there were indications that buffer conditions were also significant. This was suggestive of low specificity interactions between GSTRev and antisense RNA. Finally, specific competitor RNA was found to be unable to competitively inhibit probe binding at the concentrations used in this study. The use of a saturating GSTRev concentration may have meant that the insufficient competitor RNA was included in these reactions.

CHAPTER SIX

FUNCTIONAL RECIPROCITY OF THE REV/RRE AXIS OF OVINE/CAPRINE LENTIVIRUSES

6.1 Introduction

Chapter 5 reported the characterisation of a region within the EV1 genome (7928-8212) which could be transcribed to generate an RNA sequence able to mediate specific and high affinity binding in an *in vitro* assay to a GSTRev protein extract. A subsection of this region with co-ordinates 7995-8170 was predicted to form a highly ordered secondary structure. The coincidence of these facts suggests that the MVV EV1 Rev protein functions in a analogous manner to the more fully characterised HIV-1 Rev and HTLV-1 Rex proteins. RNA binding, however, is insufficient for full HIV Rev activity (Malim *et al.*, 1989b). The conservation of a second HIV/HTLV functional motif, the leucine-rich activation domain, and the temporal control exerted over viral gene expression by MVV (Vigne *et al.*, 1987) suggests that further aspects of Rev biology may be shared. HIV-1 Rev function has been assayed by the ability to rescue Rev-deficient, and hence replication incompetent, mutant proviruses (Pomerantz *et al.*, 1992). The effect of HIV Rev function on gene expression within the cellular environment has also been extensively investigated by the use of transient transfection experiments. Generally, expression of subgenomic constructs containing authentic viral splice sites and/or putative *cis*-acting repressive sequences (CRS) from viral structural genes is dependent on Rev function. To assay for Rev activity, expression of viral structural proteins from such constructs can be analysed. This is a more amenable procedure than viral rescue. Immune detection of Env or Gag proteins has been frequently utilised (Knight *et al.*, 1987; Hammarskjold *et al.*, 1989; Chanda *et al.*, 1990). Alternatively, reporter genes with identifiable products can be inserted within an intronic location such that the functional expression of pre-mRNA is required for detection of gene products. Defects in nucleocytoplasmic transport, stability or translation in eukaryotic cells prevent efficient expression of pre-mRNA. These may be overcome by co-expression of the Rev/RRE regulatory axis. Commonly utilised reporter genes include chloramphenicol acetyltransferase, luciferase and β-galactosidase. An alternative to assaying for protein products is to directly analyse RNA expression patterns (Chang and Sharp, 1989; Felber *et al.*, 1989; Lawrence *et al.*, 1991).

Elements which act to repress the expression of structural genes during the early phase of MVV infection have yet to be characterised. Thus, the use of reporter constructs based on HIV regions with known Rev-dependent characteristics may allow MVV Rev function

to be analysed. Insertion of the putative EV1 RRE into such vectors may place expression under EV1 Rev control, although the effect of the heterologous background would have to be considered.

Two HIV-based reporter constructs were obtained (kind gifts of Dr. B. Cullen and Dr. R. Fridell, Duke University, North Carolina). The presence or absence of Rev activity determines expression of different forms of the Tat protein from pgTAT (Malim *et al.*, 1988). This vector is responsive to the HTLV-1 Rex protein (Bohnlein, S. *et al.*, 1991), demonstrating that the HIV-derivation of this construct does not prevent the function of heterologous Rev/Rex proteins. The derivative vector pOX100 was created by substitution of the *tat* exons for the more quantifiable CAT reporter gene (Fridell *et al.*, 1993). Thus, the aim of this chapter was to investigate EV1 Rev function by cloning of the *rev* gene and the RRE element into appropriate eukaryotic vectors and the use of these vectors for transient transfection based assays. The Rev responsive properties of the putative RRE would also be revealed by these experiments.

All lentiviruses demonstrate considerable intraspecific sequence divergence. indeed, these viruses may exist in many variant forms within a single infected host organism; the term 'quasispecies' has been used to describe such variants (Holland *et al.*, 1992). Sequence variation is a consequence of the poor fidelity of the retroviral reverse transcriptase. Variation within the MVV genome has been estimated to occur at a rate of between 0.8-1.7 $x10^{-3}$ substitutions per nucleotide per year (Braun *et al.*, 1987; Querat *et al.*, 1990). The small ruminant lentiviruses (MVV and CAEV) exhibit a particularly divergent strain range (Yaniv *et al.*, 1985): the nucleotide sequences of three MVV strains contain ~20% divergence (Sargan *et al.*, 1991). This divergence is concentrated in particular genomic locations: the *rev* coding region of these three strains displays the greatest variation, with ~35% divergence at the amino acid level (Sargan *et al.*, 1991). Thus, it was of interest to discover the degree of conservation of Rev function between different ruminant lentiviruses. Detailed analysis of such conservation may aid the functional characterisation of Rev/RRE sequences important for function. Transfection assays utilised to establish the functionality of the EV1 Rev/RRE interaction could easily be adapted for such reciprocal studies by interchanging both *rev* genes and putative RRE sequences from a range of viral strains.

6.2 Rev/RRE Cloning

Although the EV1 *rev* gene and putative response element were already available in cloned form, it was decide to re-clone these regions from viral DNA so that possible variation in their origins could be minimised. Moreover, cloning from EV1 DNA would serve as a control for the attempted cloning of the homologous regions from a number of other ruminant lentiviral strains. Viral genomic DNA was obtained by extraction of DNA from virally infected ovine fibroblasts (2.4.1). The viral strains used were the MVV isolates EV1, the Dutch isoalte ZZ1050 and the Icelandic isoalte K184, and the CAEV isolate G63 (Knowles *et al.*, 1991). The CAEV Co strain molecular clone (Saltarelli *et al.*, 1990) became available at a later date. Of these strains, the genomic sequences of only EV1 and Co were known. Thus, primers for polymerase chain reaction (PCR) amplification were designed with respect to these sequences, and used in conjunction with the heterologous strains in the hope that sequence conservation would be sufficient to allow correct cloning. However, it was realised that low hybridisation efficiency due to mismatches might result in failure to isolate sequences by this approach.

6.2.1 MVV Rev Genes

The *rev* gene consists of two coding exons, separated by the length of the *env* gene. In order to isolate the *rev* gene from viral DNA, it was necessary to perform a two stage polymerase chain reaction. The primers and experimental strategy used are illustrated by figure 6.1a,b. The exons are amplified independently by the use of primers which flank each exon. The products of these reactions are then used as template for the second stage reaction. The internal primers are designed to overlap one another, such that the products of the first stage contain complementary ends. This will allow a proportion of these strands to anneal to one another during the second reaction. Priming off the 3' ends of these annealed partial duplexes results in the synthesis of a full length gene. This can then act as a template for conventional PCR amplification, primed off the external primers. This process is termed PCR mediated ligation.

Primers were designed based on the published EV1 sequence (Sargan *et al.*, 1991). The external primers, the 5' first exon and 3' second exon primers, were designed to

Figure 6.1: Cloning of MVV *Rev* Gene into the Expression Vector pBC12CMV by PCR Driven Ligation

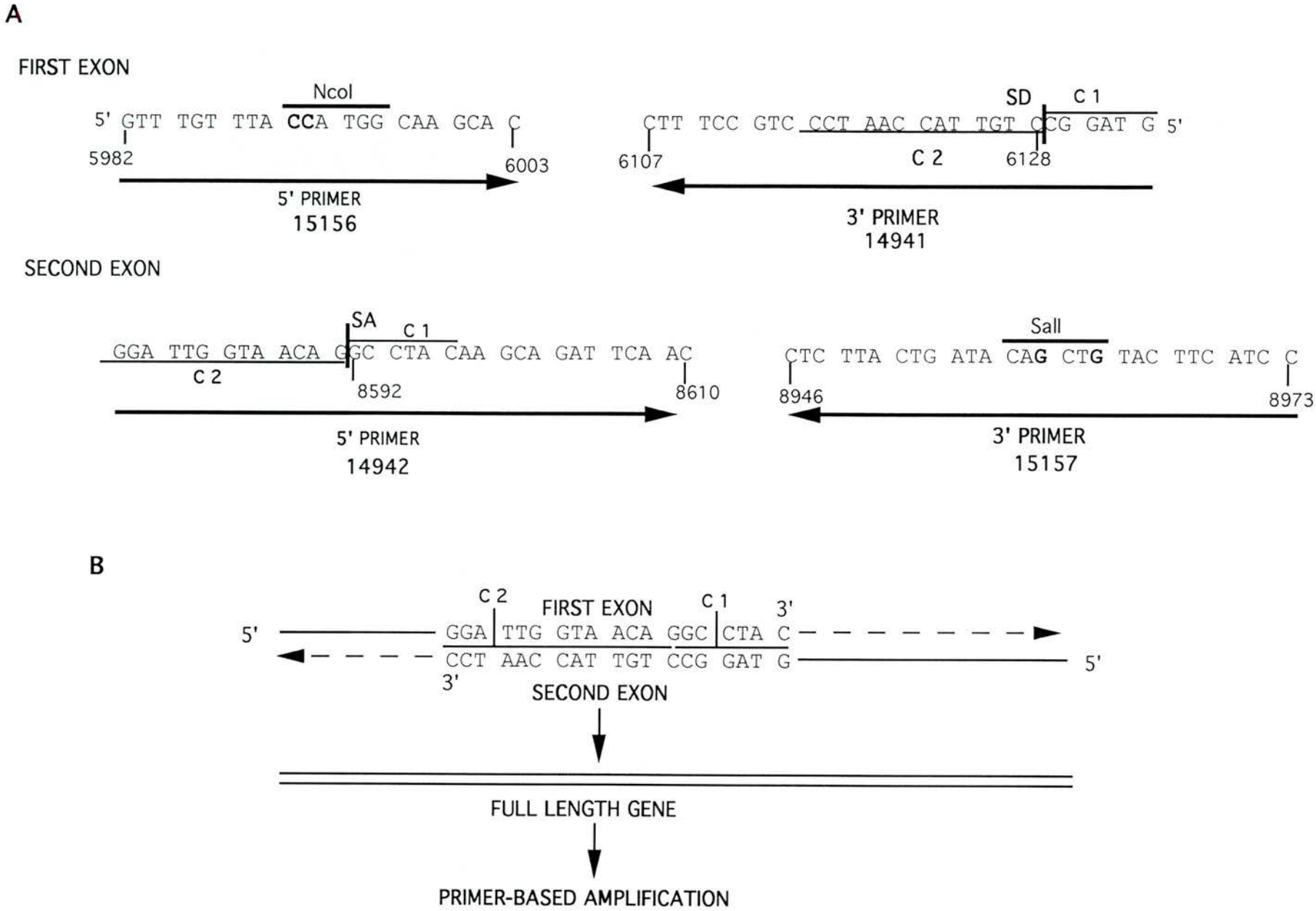
A Oligonucleotide primers for amplification of the two coding exons of the *rev* gene. The two external primers (15156 and 15157) contain mismatches with the EV1 sequence in order to incorporate specific restriction endonuclease sites into the cloned fragment. These mismatches are highlighted in **bold**. The position of the restriction sites are also shown. Numbers refer to the EV1 genomic position from which these primers were derived and to which they will hybridise. The internal primers (14941 and 14942) contain complementary sequence to allow hybridisation of the products during the second PCR reaction. These sequences are denoted C1 and C2. Thus only the 3' portion of each primer will hybridise with the location required for the first reaction, which may reduce efficiency. The EV1 *rev* splice donor (SD) and acceptor (SA) sites are marked.

B PCR driven ligation. During the second PCR reaction the 5' strand of the first exon and the 3' strand of the second exon may anneal due to the introduced complementary sequences (C1 and C2). Polymerase activity can thus be primed off the 3' end of each of these strands, to form two full length, complementary strands. Subsequent rounds of the reaction will proceed due to priming from the external oligonucleotide primers (15156 and 15157).

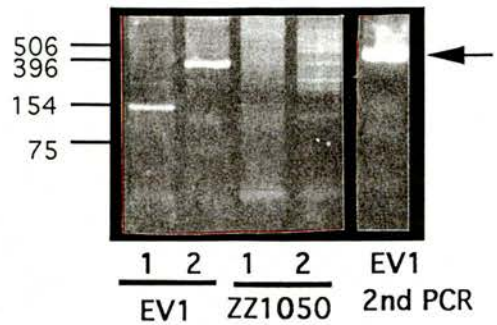
C Agarose gel electrophoresis of the first and second stage PCR products for cloning of the rev gene. The results of the first stage reaction for the first (1) and second (2) exon amplifications, from two templates, EV1 and ZZ1050 viral DNA are shown. Subsequently, the EV1 products were used as templates for the second reaction. The predicted full length gene product is marked by an arrow. Numbers refer to marker sizes in base pairs.

D The eukaryotic expression vector pBC12CMV/Rev. Expression of the inserted *rev* gene is driven by the strong, human cytomegalovirus major immediate early gene promoter (pCMV IE). The unique restriction sites NcoI and XhoI are used to insert the *rev* gene (but see text for EV1 insertion). The major portion of the plasmid is derived from pBR322, and contains an ampicillin resistance gene, and SV40 and bacterial origins of replication.

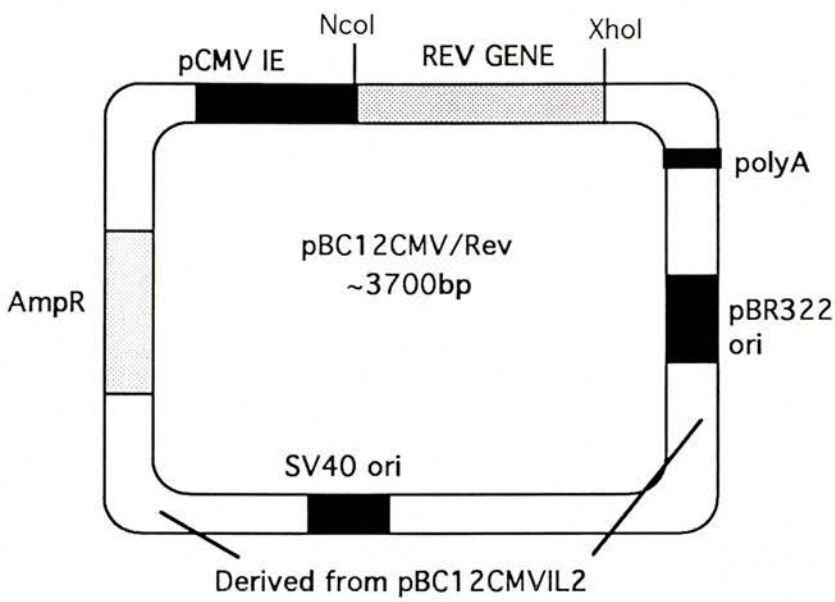
E Predicted amino acid sequence of the EV1 *rev* gene obtained by the above method. Comparison with the MVV strain 1514 Rev sequence is made. Identical amino acid residues are denoted by a dash. The exonic boundary is shown as a vertical line. Horizontal lines indicate the positions of the putative basic (EV1 residues 69-90) and activation (104-113) domains, by sequence homology with the experimentally defined HIV/SIV/HTLV domains. Translation stop codons are denoted thus (*), and gaps to accommodate sequence comparison thus (.).


A
FIRST EXON
NcoI
5' GTT TGT TTA CCA TGG CAA GCA C
5982
6003
5' PRIMER
15156
SD
C 1
CTT TCC GTC CCT AAC CAT TGT CCG GAT G 5'
C 2
6107
6128
3' PRIMER
14941
SECOND EXON
SA
C 1
GGA TTG GTA ACA GGC CTA CAA GCA GAT TCA AC
C 2
8592
8610
5' PRIMER
14942
SalI
CTC TTA CTG ATA CAG CTG TAC TTC ATC C
8946
8973
3' PRIMER
15157
B
C 2
FIRST EXON
C 1
5'
3'
GGA TTG GTA ACA GGC CTA C
CCT AAC CAT TGT CCG GAT G
3'
5'
SECOND EXON
FULL LENGTH GENE
PRIMER-BASED AMPLIFICATION

C



D



E

```
EV1     1 MASTKSKPSRATWTDMEPPQKEKWGQVVQELVTRQQNEER 40
1514    1 ---KE-----T--R-----LR-T-N--L----K---Q--E 40

       41 QGLVTGLQADSTDQIYTGNSGDRRTSGPRGKTRRRKGWFK 80
       41 EQ----S---SKA----------T-G-IG---KKKR--Y- 82

       81 WLRRLKAREKNIPAHFYPDMESNVAGLEKLTLEEKLEEKP 120
       83 ---K-R-------SQ--------MV-M-N----TQ--DNA 122

      121 IYESTTSTGGTSVAGRDWMDWRESAQK*..KRKGGLSGQR 147
      123 L-NPA-HI-DMAMD--E--E-------QKE---------- 162

        TDAHD*
      163 -N-YPGK* 169
```

incorporate restriction sites for the enzymes NcoI and SalI respectively. The internal primers contained sequence derived from either side of the *rev* splice donor and acceptor sites. Both EV1 and ZZ1050 viral DNA were used as templates for the first reaction. The expected sizes of the first stage products using EV1 DNA as template were 153nt for exon 1, and 395nt for exon two. From figure 6.1c it can be seen that products of the expected size are present in the EV1 reaction, but are absent when ZZ1050 DNA was used as the template. This was not surprising, given the high variability of the *rev* gene between sequenced MVV isolates. As discussed above, primer hybridisation to the appropriate ZZ1050 sequence was therefore likely to be weak. A similar failure to generate appropriate products was observed by PCR with the remaining viral templates. The EV1 PCR products were isolated by agarose gel electrophoresis, purified by hot phenol extraction, and used as template for the second stage reaction, using the external primers. The result of this can be seen in figure 6.1c. A product of similar size to the expected 529nt is present. This fragment was isolated as above, and digested with the restriction enzymes NcoI and SalI, to allow cloning into an expression vector.

The vector pBC12CMV was chosen to express the *rev* gene product in eukaryotic cells. This vector contains the human cytomegalovirus major immediate early gene promoter, which directs high level expression due to an active enhancer element (Cullen, 1986). It is derived from a vector designed to express interleukin 2 as an indicator of transcriptional regulation, and contains both a eukaryotic (SV40) and prokaryotic (pBR322) origin of replication, and the β-lactamase ampicillin resistance marker. The vector pcL, which contains the *rev* gene from the MVV 1514 strain (Braun *et al.*, 1987; Tiley *et al.*, 1990) as an NcoI/XhoI insert in pBC12CMV positions 67-663, was obtained (figure 6.1d: a kind gift of Dr. B. Cullen, Duke University), and for clarity, will here be here referred to as pRev1514. To obtain a vector for expression of EV1 *rev*, the 1514 fragment was removed by digestion with XhoI and NcoI. The parental vector was isolated by gel electrophoresis, excised, purified with silica and CIP digested. Ligation of the vector and the PCR-derived EV1 *rev* gene fragment to form the plasmid pRevEV1, was possible due to the compatibility of the restriction products of the enzymes XhoI and SalI (incorporation of a XhoI site into the *rev* gene fragment by PCR was contraindicated by the presence of such a site at position 8658 in the EV1 genome, within the *rev* second exon). Ligated DNA was transformed into DH5α bacterial cells, and approximately twenty colonies were obtained. These were screened for possession of the insert by restriction digest analysis. Colonies containing the expected insert were used to

generate large scale DNA preparations, and the correct insertion of the gene fragment validated by double stranded sequencing. Primers for sequencing were designed to hybridise with vector sequence approximately 50bp distant from the site of cloning. The sequence of these primers (5'-3') and the positions within pBC12CMV from which they were derived, were as follows:

5' sequencing primer G6711: CGT GTA CGG TGG GAG GTC TA: pBC12CMV 3885-3904
3' sequencing primer G6712: GCA GCA CTG ATC CAC GAT G: pBC12CMV 714-732

A single base change from the published EV1 genomic sequence, at *rev* gene position 32 (genomic position 6025) was identified. Interestingly, this change, a cytidine for thymidine substitution, was also demonstrated by pRev5, the fragment used to construct the Ty and pGEX expression vectors (3.2.2). This substitution results in an alanine for valine change in the amino acid sequence.

An amino acid sequence comparison between the predicted Rev proteins encoded by the vectors pRev1514 and pRevEV1 is presented in Figure 6.1e. The overall sequence identity between these proteins is 65.3%. Many of the non-identical residues represent conservative changes. This is especially true within the two putative functional domains. The 1514 protein has a bias towards lysines within the basic domain, compared to the arginine bias demonstrated by EV1. This suggests that basic nature, rather than specific amino acid identity may be important in mediating RNA binding. Within the leucine-rich domain, there are conserved hydrophobic residues in identical positions in each protein.The EV1 protein is truncated with respect to the 1514 protein (147 residues compared to 169 residues). This is a consequence of the presence of a translation termination codon at the 3' terminus of the *rev* gene. The conservation of the predicted sequence downstream of this codon with that expressed by 1514 suggests a recent, secondary mutation event has ablated expression of this region in EV1.

6.2.2 Rev Responsive Elements

Data obtained from the *in vitro* RNA binding studies presented in chapter 5, and from reports of the Rev activity of the MVV 1514 strain and of a potential RRE within the CAEV CO

strain *env* gene (Tiley *et al.*, 1990; Saltarelli *et al.*, 1990) suggested that the ovine/caprine lentiviruses possess a Rev responsive element in a conserved genomic location. To isolate the RRE from genomic DNA, primers were designed to flank the putative element, and were centred on sequence which allowed the incorporation of restriction sites with the minimum of nucleotide substitution. A 5' primer, 14949, included a recognition site for BglII, and allowed ligation into both reporter constructs. However, differences in the cloning regions of these vectors meant that distinct 3' primers were required, and that two independent cloning reactions were necessary. Isolation by PCR and subsequent cloning of the EV1 RRE fragment by the 14949 and G0242 primers has been discussed (5.3.3). This fragment was used for cloning into the vector pOX100pL (see below). To amplify fragments for insertion into the reporter vector pgTAT, a primer incorporating a HindIII restriction site was synthesised (14948: figure 6.2a). Amplification with 14949 and 14948 predicts a 337nt product, encompassing EV1 genomic sequences 7920-8256. The results of PCR using these primers and the various virally-infected cell derived DNA is presented in figure 6.2c. All four templates give rise to a product of the expected size, although in the ZZ1050 reaction this is not the major product, and only in the EV1 reaction is this the only visible product. These fragments were excised from an agarose gel, purified and cloned directly into pgTAT by digestion with HindIII and BglII, with subsequent ligation into a similarly restricted pgTAT plasmid. Sequencing of plasmids derived from transformants was used to confirm the nature of the insert. Primers for double stranded sequencing were designed to hybridise with regions of the pgTAT plasmid approximately 30 base pairs distant from either cloning site. The sequence and hybridisation positions of these primers was as follows (5'-3'):

5' primer G0192: GGC CAG TAG TAT CAA CTC pgTAT 1949-1966.

3' primer G0191: CTT TTC TTG CTG GTT TTG CG pgTAT 3160-3141.

The sequence obtained confirmed the correct cloning of the EV1 sequence, which was identical to the published sequence except for the inclusion of four nucleotide changes identical to those described in 5.3.3. This was expected, due to the use of the same template DNA for cloning of these two fragments. However, the inserts cloned from the remaining viruses did not appear to contain viral-derived sequence. Despite the amplification of a fragment of the expected size by PCR this was not entirely unexpected, given the probable

Figure 6.2: Cloning of the Putative Rev Responsive Elements from Ungulate Lentiviruses.

A Oligonucleotide primers for PCR amplification of the RRE from various ungulate lentivirus strains. Primers were designed from the known EV1 sequence. Numbers refer to the predicted genomic hybridisation positions with respect to the EV1 sequence. The positions of incorporated restriction sites are shown, as are the base mismatches introduced to accommodate these sites (**bold**).

B Oligonucleotide primers for PCR amplification of the CAEV CO strain RRE. Primers were designed from the known CO sequence. Other information as above (A).

C Agarose gel electrophoresis of products of the PCR using primers 14949 and 14948 (A) for amplification of the RRE. The arrow indicates the predicted correct product. The templates for PCR are shown below. MVV strains, EV1, ZZ1050 (ZZ) and K184, and the CAEV strain, G63.

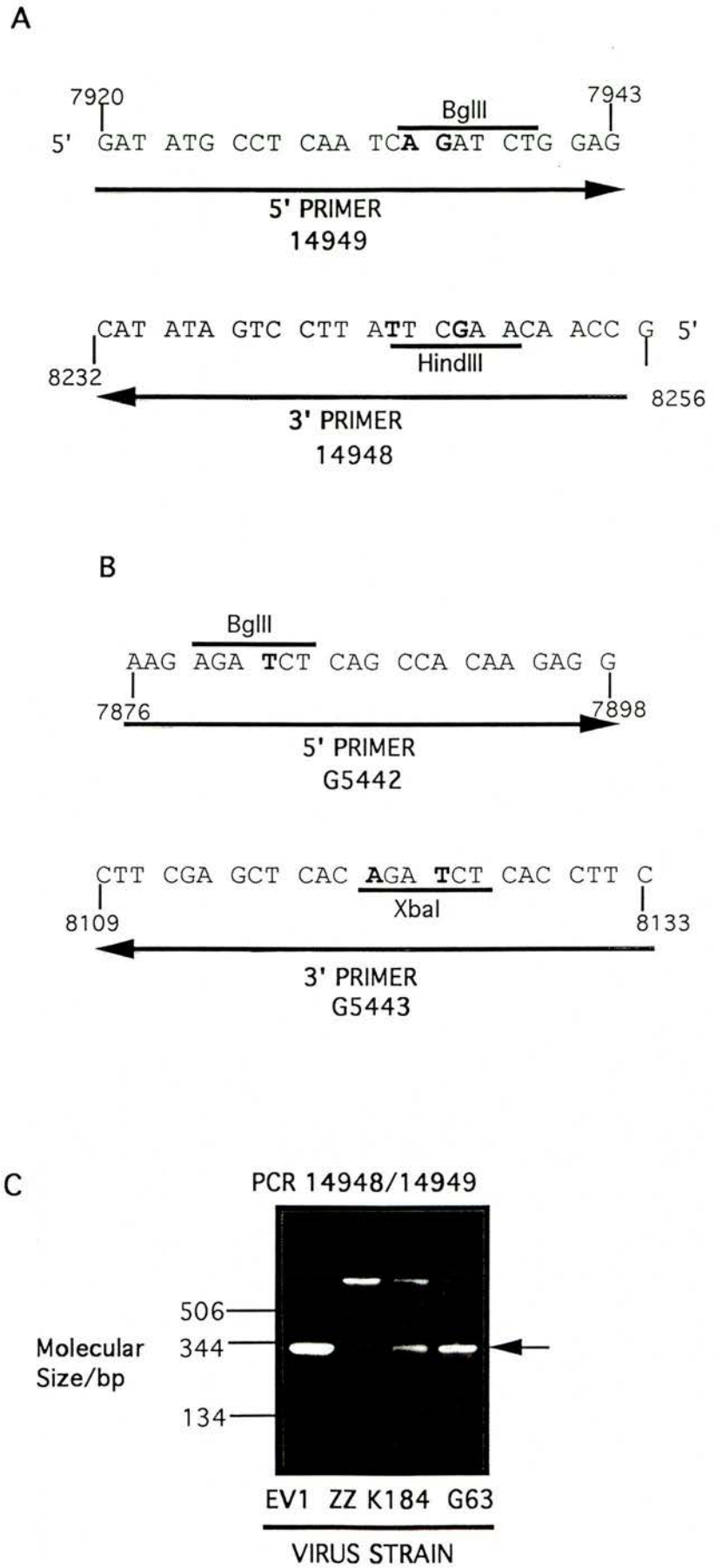

A
7920
7943
BglII
5' GAT ATG CCT CAA TCA GAT CTG GAG
5' PRIMER
14949
CAT ATA GTC CTT ATT CGA ACA ACC G 5'
HindIII
8232
8256
3' PRIMER
14948
B
BglII
AAG AGA TCT CAG CCA CAA GAG G
7876
7898
5' PRIMER
G5442
CTT CGA GCT CAC AGA TCT CAC CTT C
XbaI
8109
8133
3' PRIMER
G5443
C
PCR 14948/14949
506
344
134
Molecular
Size/bp
EV1 ZZ K184 G63
VIRUS STRAIN

Figure 6.3: Primary and Secondary Structure of the Cloned Ungulate Lentiviral Putative RREs

A Nucleotide sequence comparison of the region with predicted higher order structure from the cloned RREs. Identical nucleotides to the 1514 sequence are denoted thus (.). Positions 1-200 correspond to genomic positions 7923-8122 (1514: Braun *et al.*, 1987), 7985-8184 (EV1: Sargan *et al.*, 1991) and 7907-8106 (CAEV CO: Saltarelli *et al.*, 1990). The SU/TM Env boundary occurs from the G at position 2 in all these viruses.

B Percentage primary sequence divergence between the above regions of EV1, 1514 and CO.

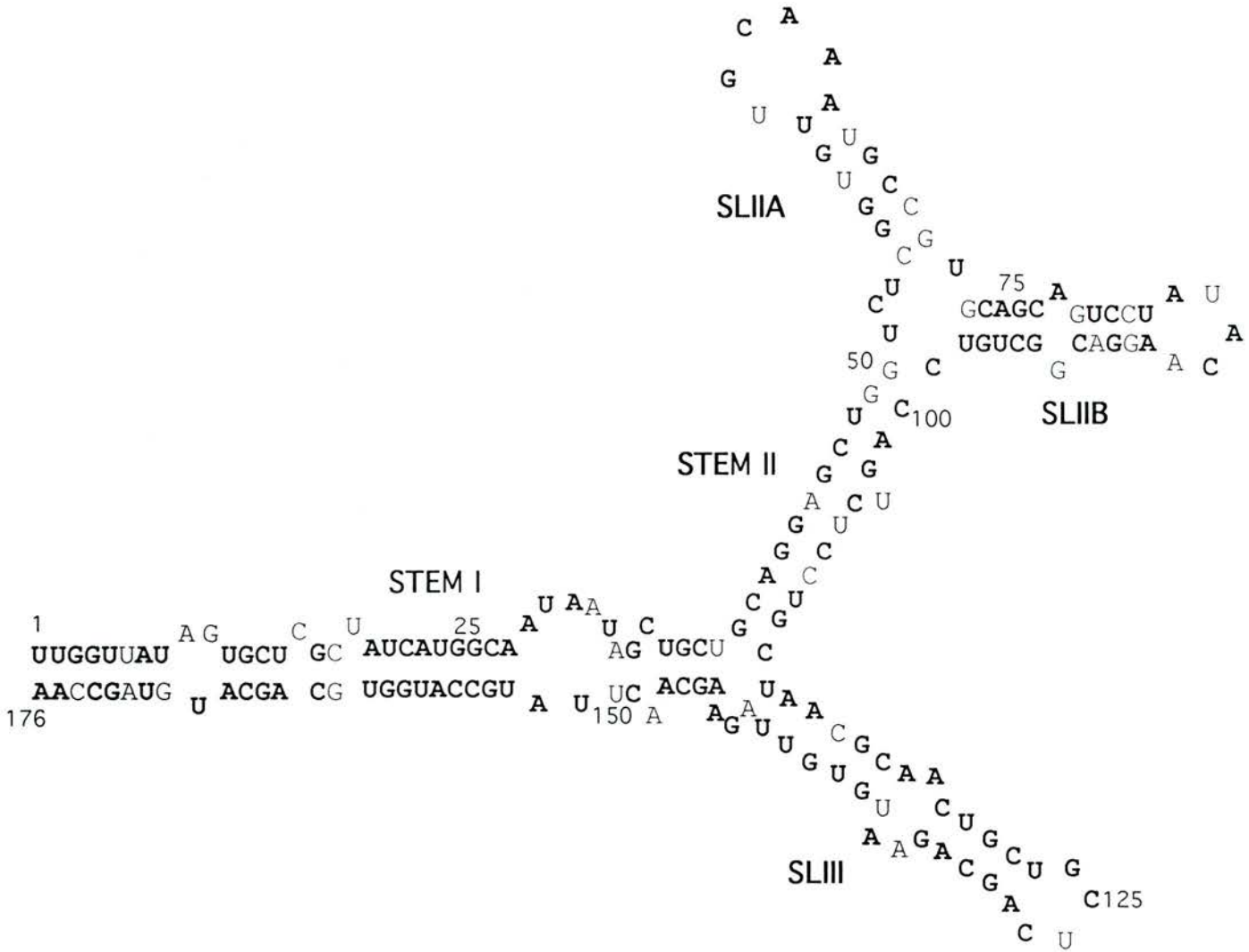
C Representation of the predicted secondary structure of an RNA element derived from the above sequences. The structure was predicted using the EV1 sequence; those bases which are conserved between all three viruses are highlighted in **bold**. Substructures are named by analogy with the HIV-1 RRE. Position 1 corresponds to position 10 in the above sequence comparison. Prediction was made using the UWGCG (version 8) programme 'FOLDRNA'.

A

```
MVV 1514
MVV EV1
CAEV CO


1
AGGGATAGGC TTGGTTATTG TGCTAGCCAT CATGGCAATA ATCGCTGCTG
G..C.....G ........A. ....C..T.. .......... ..A.......
...CG.T... .....C...A ......TT.. .......... G.A.....C.

51
CAGGAGCTGG TCTCGGTGTC GCAAACGCCG TGCAGCAATC CTATACCAGG
.......... .........T .....T.... .......G.. ......A...
....G...TC ...G..A... ........GA .T.....G.. T..C..T.A.

101
ACGGCTGTCC AGTCTCTTGC TAACGCAACT GCTGCCCAGC AGGAAGTGTT
.......... ......C... .......... .....T.... ..A.T.....
G.A....... ..A.C..... ...T...... .....A.... ....T.....

151
GGAAGCATCG TATGCCATGG TACAGCATAT AGCCAAAGGA ATAAGAATCC
A......A.T .......... .G......G. .......... G.......AT
A..G...A.C .......... ........G. G..T.....C G..C.....T
```

B Percentage Sequence Divergence of Putative RREs

	CO	EV1
1514	20.5	11.5
EV1	20.5	


SLIIA
SLIIB
STEM II
STEM I
SLIII
1
25
50
75
100
125
150
176
UUGGUUAU
UGCU
GC
AUCAUGGCA
AACCGAUG
ACGA
CG
UGGUACCGU
GCAGC
GUCCU
UGUCG
CAGGA

degree of variation between the sequences of these viruses. The plasmids containing these viruses were discarded, and unfortunately time did not allow for a further attempt to clone the RREs by more stringent PCR or by other methods. However, subsequent to the completion of the pgTAT assay section of this report, a molecular clone and the entire genomic sequence of the CAEV Co strain (Saltarelli *et al.*, 1990) became available. Primers for amplification of the putative RRE were designed (figure 6.2b), and a fragment of the expected size (258bp) obtained. This fragment was subcloned into the pCRII vector of the TA cloning kit, and subsequently ligated into pOX100/pL after restriction with BglII and XbaI.

The sequence of the 200 bases encompassing the putative RREs of the viruses used in this study, MVV EV1, 1514 and CAEV Co are compared in figure 6.3. The nucleotide primary sequence is illustrated by figure 6.3a, the percentage dissimilarity of this region by figure 6.3b, and the predicted secondary structure by figure 6.3c. The two Maedi Visna viral isolates demonstrate a greater degree of similarity to one another than to the CAEV isolate from which they are equally diverged. Conserved bases are distributed throughout the predicted secondary structure element. However, several regions, such as the length of steml display particularly strong conservation. This is presumably due to the requirement to maintain base pairing within this region. Primary sequence variation is generally structurally conservative, so that essentially the same structure is predicted for all three sequences. Some variation in the content and position of substructures such as bulges is seen. Minor variations in predicted formation energies are also recorded.

6.3 Vectors for Functional Assays

6.3.1 pgTAT

The assay vector pgTAT contains a section of the genome of the HIV-1 HXB-3 proviral clone within the pBC12CMV background (Malim *et al.*, 1988). Both exons of the *tat* gene are present within the HIV-1 insert. These are separated by an intron which comprises the 5' and central regions of the *env* gene, and includes the RRE (figure 6.4a). The intron is delineated by authentic HIV-1 splice sites, and may contain *cis*-acting repressor sequences within the *env* coding region. Thus, expression of pgTAT is dependent on Rev activity. In the absence of Rev activity, this vector predominantly expresses a fully spliced cytoplasmic *tat* mRNA, which gives rise to the two-exon, 86 amino acid form of Tat. However, if Rev activity is present,

then splicing can be avoided, and a substantial proportion of the RNA is present in the cytoplasm in an unspliced form. This is translated to produce the single exon (72 amino acid) form of Tat. The pattern of Tat expression can be assessed by immunoprecipitation of cell extracts with an anti-Tat antiserum and subsequent electrophoretic separation. No other HIV proteins are expressed by this vector (Malim *et al.*, 1988). The HIV-1 RRE in pgTAT is flanked by unique restriction sites, for BglII and HindIII. Excision of the RRE by digestion at these sites allows the insertion of heterologous sequence. These can therefore be assayed for RRE-activity. The vector pgTAT/RRE-V contains the MVV 1514 putative RRE sequence (genomic positions 7923-8124) (Tiley and Cullen, 1992). This vector is responsive to the Rev expression plasmid, pRev1514.

6.3.2 pOX100/pL

A second reporter construct was available. This vector is a hybrid constructed by replacement of the first *tat* coding exon of pgTAT with an insert derived from the vector pDM128 (figure 6.5) (Hope *et al.*, 1991b). The pDM128 plasmid contains a 780 base pair chloramphenicol acetyltransferase gene within the env coding region of the HIV-1_{SF2} proviral clone. Definition of the CAT/*env* region as an intron by the presence of flanking HIV-1 splice sites and the presence of the RRE means that CAT gene expression is under Rev mediated control. The hybrid pOX100/pL was constructed by excision of the splice donor/CAT region from pDM128 and its insertion into SalI/BglII restricted pgTAT. The intronic location of the CAT gene is maintained by the presence of the splice acceptor site within pgTAT. A polylinker sequence has been engineered in place of the HIV-1 RRE, and allows insertion of heterologous response elements. A pOX100pL derivative vector, pOX100/1514, was also obtained (a kind gift of Dr. R Fridell, Duke University). This construct contains the 1514 RRE (genomic positions 7901-8127) ligated into the BglII/XbaI site of pOX100/pL. The EV1 fragment generated by PCR with the primers 14949 and G0242 (5.3.3) and cloned into the pCRII vector was released by digestion with BglII and XbaI and ligated into a similarly restricted pOX100/pL vector. Subcloning was confirmed by restriction digest analysis.

6.4 Rev Functional Assays

6.4.1 Transient Transfection

COS cells (Gluzman *et al.*, 1982) are SV40 transformed African green monkey kidney cells which constitutively express the large T antigen. Transfection of these cells by chemical treatment of cells which results in DNA interaction with cell membranes, and possible DNA entry by endocytosis proceeds relatively efficiently. Although only a proportion (10-20%) of the cells are competent for transfection, copy number per cell may be high (Cullen, 1987). Transfected plasmids are expressed at peak levels for 48-72 hours post-transfection. COS cells were initially transfected by DEAE-dextran treatment, the reported optimal method for this cell line (Cullen, 1987). Subsequent analysis of transfection efficiency, important to reduce inter-assay variation with the quantifiable CAT assay, suggested that the calcium phosphate precipitation method was more efficient for this particular cell line. Throughout this work, Qiagen column chromatography was used for the purification of plasmids, as the quality of DNA preparation may adversely affect transfection efficiency. Standard amounts of plasmid were transfected (5μg per 35mm dish for calcium phosphate transfection), using the parental vector pBC12CMV to maintain this amount where necessary.

6.4.2 Radioimmunoprecipitation: pgTAT

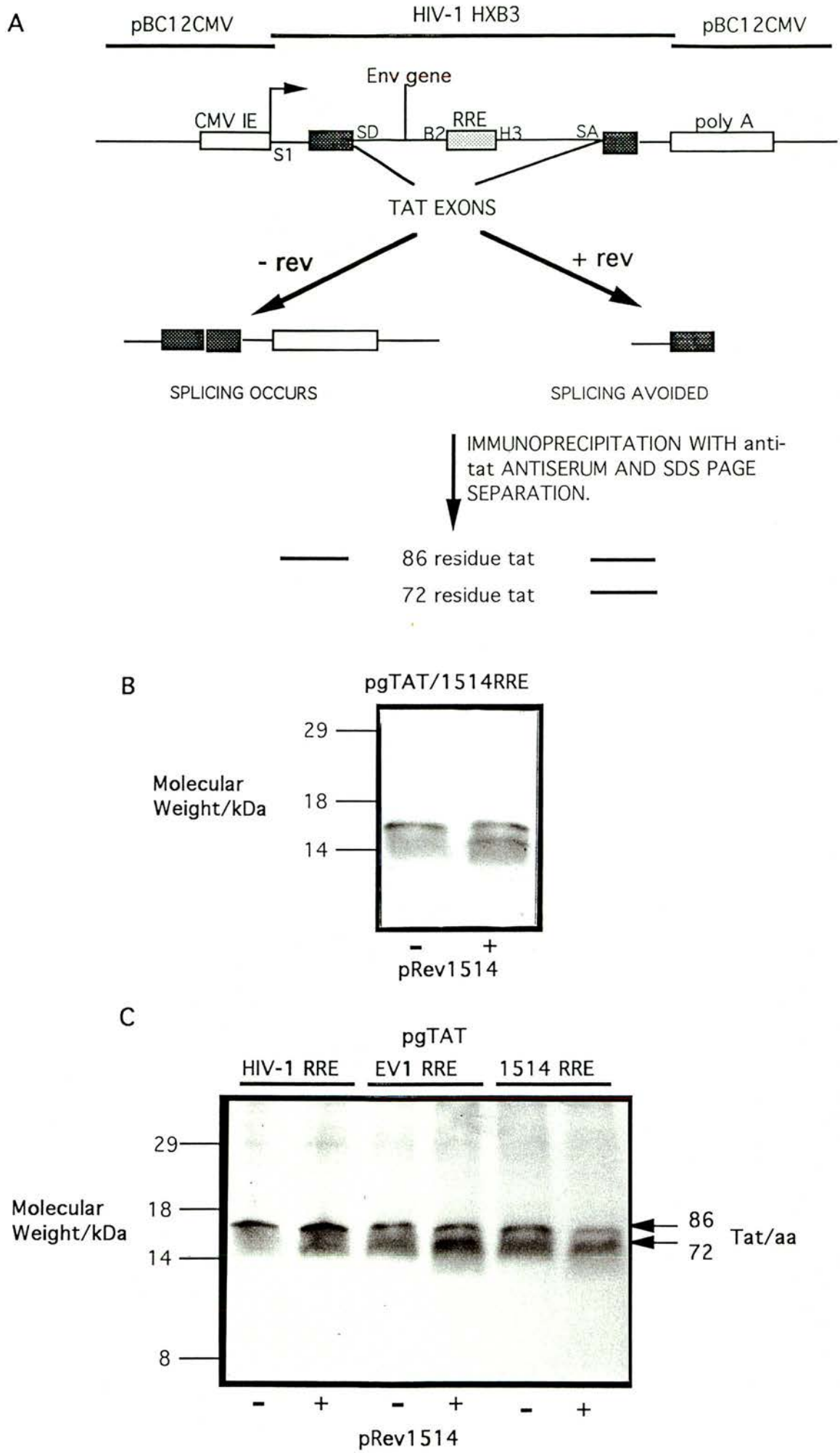
COS cells were grown to near confluence in 35mm six-well plates, and transfected by the DEAE-dextran method (2.20.3). Each plasmid, the indicator construct pgTAT and derivatives, and the expression construct pBC12CMV and derivatives, was added in a 500ng aliquot, with the total DNA content standardised at 1μg. An initial assay to test the protocol was performed using 500ng each of pRev1514 and pgTAT/vRRE, which are both derived from the MVV 1514 strain, and have been shown to provide a positive result in this assay (Tiley *et al.*, 1991). Transfected cells were labelled with tran^{35}S label after 48 hours incubation, and subsequently harvested. Immunoprecipitation with an anti-Tat antibody (a kind gift of Dr. B Cullen, Duke University) was followed by electrophoretic resolution through a 15% SDS-PAGE gel. The result was visualised by autoradiography, and is illustrated by figure 6.4b. In the presence of the parental vector pBC12CMV alone, pgTAT/vRRE gives rise to a single form of Tat of ~16kDa apparent molecular weight. Co-transfection with pRev1514

Figure 6.4: The pgTAT Radioimmunoprecipitation Assay

A The reporter construct pgTAT. Regions derived from the HIV-1 HXB3 (genomic sequence 5367-8055) proviral clone, and the pBC12CMV parental vector are indicated. Tat exons are shown by dark stipling. SD: splice donor site, SA: splice acceptor site. Restriction sites: SI - SalI, B2 - BglII, H3 - HindIII. A schematic outline of the assay procedure is shown.

B Results of preliminary pgTAT assay. COS cells were co-transfected with pgTAT/1514RRE and pRev1514 or the parental vector pBC12CMV. Cells were labelled with ^{35}S amino acids after 48 hours and harvested. Tat protein was immunoprecipitated with anti-Tat antiserum and resolved by electrophoresis through a 15% polyacrylamide gel. Gels were dried and the results visualised by autoradiography.

C pRev1514 was co-transformed with the original pgTAT plasmid (+HIV-1 RRE) and the MVV derived pgTAT/EV1RRE and pgTAT/1514RRE. Assays were performed as above (B). Arrows indicate the position of the two forms of Tat, the 86 amino acid two-exon and the 72 amino acid one exon form.


A
pBC12CMV
HIV-1 HXB3
pBC12CMV
Env gene
CMV IE
S1
SD
B2
RRE
H3
SA
poly A
TAT EXONS
- rev
+ rev
SPLICING OCCURS
SPLICING AVOIDED
IMMUNOPRECIPITATION WITH anti-tat ANTISERUM AND SDS PAGE SEPARATION.
86 residue tat
72 residue tat
B
pgTAT/1514RRE
Molecular Weight/kDa
29
18
14
-
+
pRev1514
C
pgTAT
HIV-1 RRE
EV1 RRE
1514 RRE
Molecular Weight/kDa
29
18
14
8
86
72
Tat/aa
-
+
-
+
-
+
pRev1514

induces the expression of a second form of Tat, with an apparent molecular weight of 14-15kDa. Thus, the outcome of expression of pgTAT/vRRE is dependent on the presence of functional Rev activity. A second experiment was performed in order to investigate the functional compatibility of pRev1514 with the RRE of other viruses. This result is illustrated by figure 6.4c. The original pgTAT vector, containing the HIV-1 RRE, is unaffected by pRev1514 expression, generating only a single Tat protein form. Both the 1514 and EV1-RRE containing pgTAT vectors appear to be responsive to pRev1514 expression. However, it is clear that the relatively poor resolution of this autoradiograph makes a definite conclusion difficult. Further experiments produced essentially similar results, with poor resolution of the bands due to Tat protein. Electrophoresis through a 10-20% gradient gel did not result in more effective resolution, nor did immunoprecipitation with protein A sepharose in place of agarose. Replacing the tran^{35}S label with pure ^{35}S labelled methionine also proved ineffective. Thus, interpretation of the results achieved with this assay was considered to be unreliable, and further analysis was not carried out.

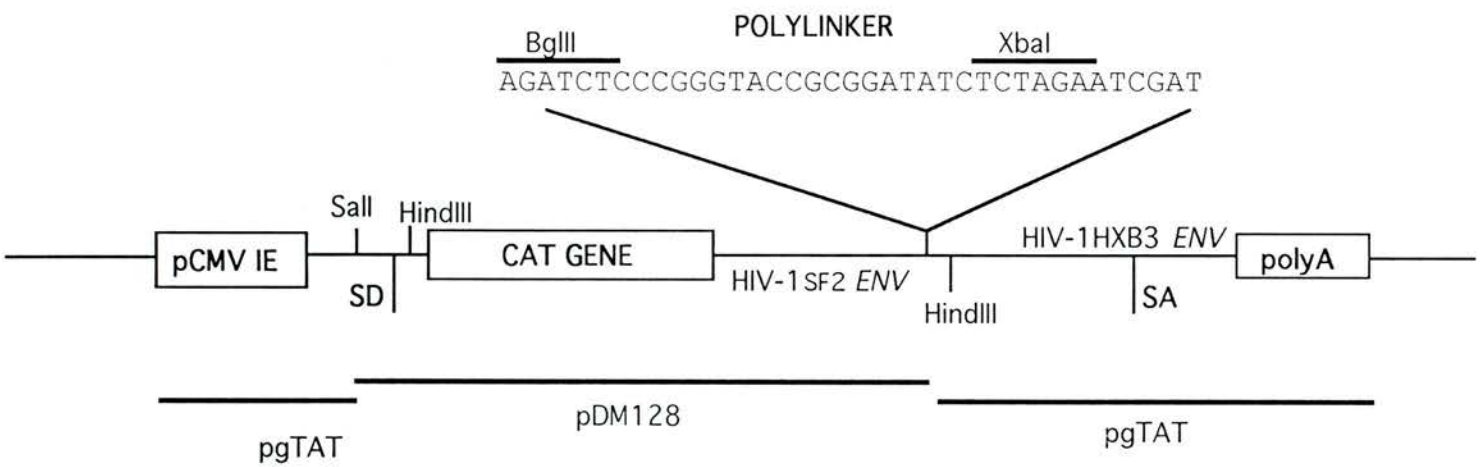
6.5 CAT Assays

6.5.1 Introduction

The bacterial drug resistance gene, chloramphenicol acetyltransferase (CAT), is the most widely used reporter gene for transient transfection assays (Gorman *et al.*, 1982; Cullen, 1987). Although commonly used as an indicator of promoter strength, the CAT assay can be adapted to investigate other aspects of gene regulation. The level of expression of a construct containing the CAT gene can be directly related to the resulting CAT enzymatic activity. Measurement of CAT activity is a rapid and sensitive technique, with the added advantage of low background readings due to the restriction of this gene to prokaryotes. CAT catalyses the inactivation of chloramphenicol by acetylation of hydroxyl groups (Shaw, 1975). Methods to detect CAT activity take advantage of the differential solubility of substrate and product. Thus, chloramphenicol is soluble in aqueous solution. However, acetylation increases hydrophobicity, resulting in ready substrate solubility in organic solvent. This divergence between substrate and product is increased if the acyl group is derived from the co-factor, n-butyryl Co-enzyme A. Thus, discrimination between product and substrate is more efficient, and the two can be separated by a phase extraction technique (Seed and

Figure 6.5: The Reporter Construct pOX100/pL

The important functional regions of pOX100/pL are shown. Sequence originating from the pgTAT parental vector, and the pDM128 insert are indicated. The nucleotide sequence of the polylinker region is shown in detail, this region replaces the HIV-1 RRE. The positions of the two restriction sites used in this report for subcloning are highlighted. The CAT gene is inserted into a background derived from HIV-1 clones, and is defined as an intron by the presence of authentic HIV splice donor (SD) and acceptor (SA) sites.


POLYLINKER
BglII
XbaI
AGATCTCCCGGGTACCGCGGATATCTCTAGAATCGAT
SalI
HindIII
pCMV IE
SD
CAT GENE
HIV-1SF2 ENV
HindIII
HIV-1HXB3 ENV
SA
polyA
pgTAT
pDM128
pgTAT

Sheen, 1988). The use of back-extractions meant that only ~0.035% of the substrate chloramphenicol was detected in the organic phase during the development of this technique (Seed and Sheen, 1988). The phase extraction technique has a further advantage over other techniques in that it generates linear results over a two to three order of magnitude enzyme concentration. The use of ^{14}C labelled chloramphenicol allows the assay to be scored by scintillation counting. As the presence of certain detergents may inhibit CAT activity, cell extracts were prepared by three rounds of freeze/thawing. In this chapter, results are presented as the percentage of input ^{14}C-chloramphenicol converted to butyrylated product in each extract.

6.5.2 Assay Parameters

6.5.2.1 Transfection Efficiency

The quantifiable nature of the CAT assay meant that experiments were required to be controlled for variability in transfection efficiency and cell extract preparation. The plasmid pSV-β-galactosidase (Promega) was used as a control. This expresses the enzyme β-galactosidase under the control of the SV40 promoter. It must be assumed that the control and experimental plasmids are transfected and expressed identically. A standard amount (1μg) of this plasmid was co-transfected with assay vectors. After harvesting, the cell lysate (10μl) was tested for β-galactosidase activity by a colorimetric assay (2.22.3) and the absorbance at 420nm measured by spectrophotometry. To obtain standard values, the β-gal activity was translated into units of enzyme by reference to a standard curve derived from known concentrations of purified enzyme. Individual cell extracts were then standardised for transfection/extraction efficiency by use of a correction factor to convert to a standard β-Gal activity.

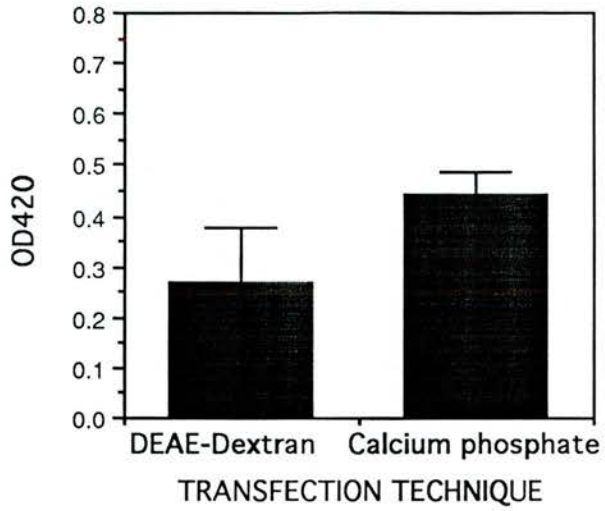
Transfection with the pSV-β-Gal control plasmid was used to judge the relative efficiencies of transfection using the DEAE-dextran and calcium phosphate precipitation techniques. It was noted during preliminary investigation that the COS cell line used appeared sensitive to DEAE-dextran treatment, and that cell death resulted in variability in the volume of cell extract obtained. Thus, the pSV-β-Gal plasmid (1μg) was transfected into COS cells in four 35mm dishes by both techniques, and the resulting β-gal activity compared. From figure 6.6a

Figure 6.6: CAT Assay Parameters

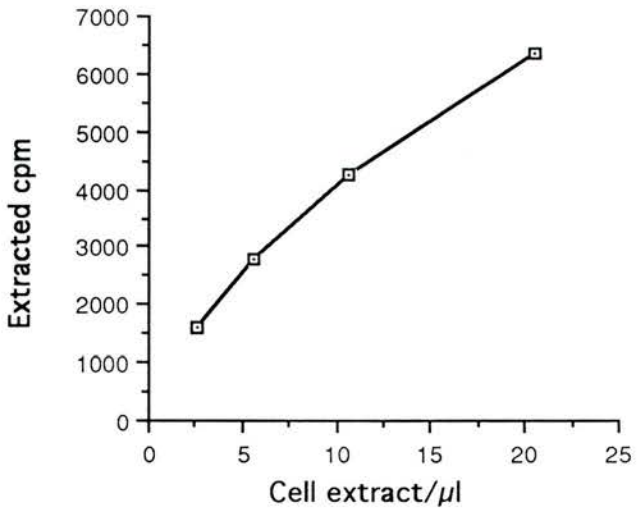
A The relative efficiency of transfection techniques. COS cells were transfected with the control plasmid pSV-β-Gal by DEAE-dextran or calcium phosphate precipitation. Cells were harvested after incubation for 48 hours, and a cell lysate prepared by three rounds of freeze-thawing. B-gal activity was assayed by standard techniques and is presented as absorbance at 420nm after one hour incubation. The results are shown as the mean +/- standard deviation from four transfections. The results differ from one another significantly ($p<0.05$). Statistical analysis using two sample t test for independent variables.

B / C Variation of parameters during a standard CAT assay. Assay was performed using an extract prepared from cells co-transfected with the plasmids pRevEV1 and pOX/EV1RRE. CAT assay was carried out with variation in either the volume of extract used (B) or the incubation time before reaction termination (C). Results are presented as the amount of xylene extracted ^{14}C-butyrylated chloramphenicol (cpm).

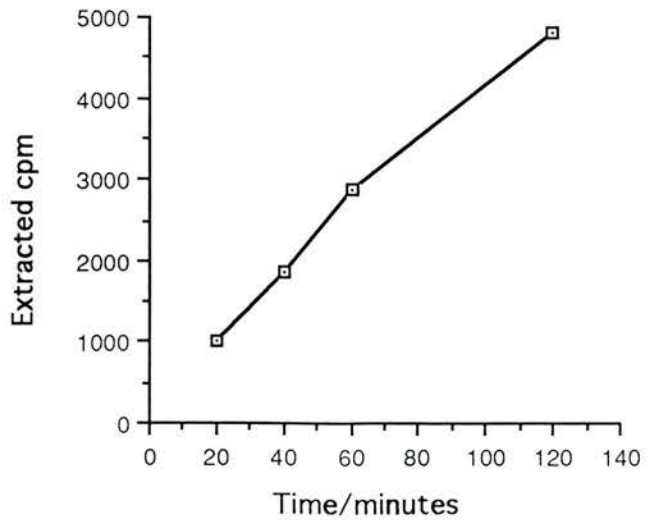
A


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
OD420
DEAE-Dextran
Calcium phosphate
TRANSFECTION TECHNIQUE

B


7000
6000
5000
4000
3000
2000
1000
0
Extracted cpm
0
5
10
15
20
25
Cell extract/μl

C


5000
4000
3000
2000
1000
0
Extracted cpm
0
20
40
60
80
100
120
140
Time/minutes

it can be seen that calcium phosphate precipitation gives rise to a higher enzyme activity; this proved to be a significant difference ($P<0.05$). Thus, calcium phosphate precipitation was used for transfection of both expression and reporter constructs for further analysis.

6.5.2.2 CAT Assay Parameters

The linearity of the results of the CAT assay with respect to enzyme concentration and activity, and hence to the degree of gene expression, was investigated by analysis of extracts prepared from cells co-transfected with pRevEV1 and pOX/EV1, which results in substantial CAT activity (see below). The phase extraction assay was performed using a range of cell extract volumes (figure 6.6b) or by using a single volume (5µl) and varying the reaction time before extraction (figure 6.6c). As can be seen from these results, the recorded CAT activity is effectively linear over the ranges used. Thus the phase extraction assay was deemed an appropriate technique for these studies, and could be used without extract dilution. CAT activity from cells transfected with heterologous vectors, such as pRev1514, was found to be very low (% acetylation <0.01% of input chloramphenicol) which suggested that the phase extraction procedure was an effective discriminatory method between substrate and product. For further analysis, the background level of CAT activity was assumed to be zero. Statistical analysis was carried out using a standard two sample t-test for independent variables.

6.5.3 Basal CAT Activity

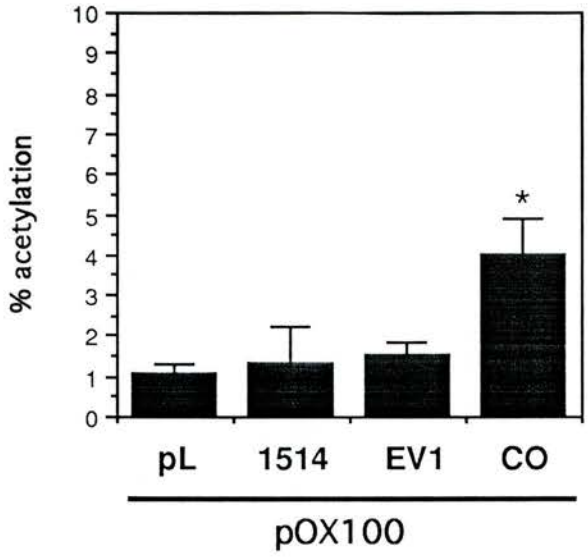
It has been reported that the removal of the HIV-1 RRE from vector constructs may lead to an increase in the basal level of expression (Huang *et al.*, 1991). This observation has been suggested to be the consequence of the presence of a *cis*-acting repressor sequence (CRS) overlapping the RRE (Brighty and Rosenberg, 1994; Nasioulas *et al.*, 1994). Partial or complete removal of this element would thus relieve the block to gene expression, as has been demonstrated by inactivation of a *gag* gene CRS (Schwartz *et al.*, 1992). In order to investigate the possible presence of a similar repressive element within the RRE-containing gene sequences of the ungulate lentiviruses, the basal level of activity of each of the pOX100 derivatives was analysed. Data was obtained from three independent transfections, and is presented by figure 6.7a. A low level of CAT enzyme activity (~1% acetylation with these

Figure 6.7: Basal Expression from pOX100/pL and Derivative Vectors

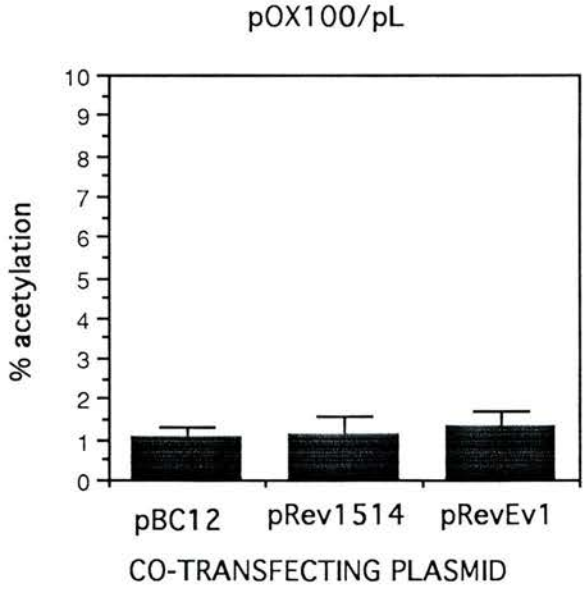
A COS cells were transformed with pOX100/pL (2μg) or derivative vectors as indicated by calcium phosphate precipitation, and extracts prepared after 48 hours. An aliquot of extract (5μl) was assayed for CAT activity, and the results presented as the percentage of input ^{14}C chloramphenicol converted to the butyrylated form, after correction for transfection/extraction efficiency by β–Gal activity. Results shown are the average of three independent transfections (mean +/- standard deviation). * indicates result significantly different ($p<0.01$) from the parental vector pOX100/pL.

B The effect of co-transfection of pOX100/pL with pBC12CMV and derivative Rev expression vectors. Assay procedure and result presentation as described above (A).

A


% acetylation
10
9
8
7
6
5
4
3
2
1
0
*
pL
1514
EV1
CO
pOX100

B


pOX100/pL
% acetylation
10
9
8
7
6
5
4
3
2
1
0
pBC12
pRev1514
pRevEv1
CO-TRANSFECTING PLASMID

assay parameters) was detected by transfection with the pOX100 plasmid alone. This level presumably reflects inefficient nucleocytoplasmic transport and translation of CAT pre-mRNA in the absence of Rev. Insertion of the EV1 and 1514 RREs did not alter the basal level of CAT activity. However, insertion of the CAEV CO RRE resulted in an approximate 4 fold increase in basal activity. The increase in acetylated product over that generated by pOX100/pL proved to be significant ($P<0.01$). Thus, the CO RRE has the opposite effect to that subscribed by some researchers to the HIV-1 RRE. This is a surprising finding, especially given the degree of sequence and structural identity between the CO and 1514/EV1 RREs. Hypothetically, this could be due to the possession of a positive enhancing effect on expression by the inserted sequence, or to a negative effect on surrounding repressor sequences. This latter option appears to be more likely. It is possible that the conformation of the CO RRE may interfere with regions of the pOX100 pre-mRNA which mediate reduced expression, perhaps through nuclear retention (see 1.14.2). The lack of a similar effect due to the isosteric MVV RREs may contradict this; however, the inserted regions are not identical in size or genomic location and include RRE flanking sequences.

The effect of co-transfection of the pBC12CMV-based expression vectors pRev1514 and pRevEV1 on CAT expression from the parental vector pOX100/pL was investigated. From figure 6.7b it can be seen that the level of CAT activity is unaffected by co-transfection with pBC12CMV or its Rev-expressing derivatives. Thus, MVV Rev is unable to activate the expression of this construct in the absence of putative responsive elements.

6.5.4 The Effect of Rev Co-Expression on CAT Activity

To study the effect of Rev expression on indicator constructs containing putative RRE inserts, COS cells were co-transfected with pOX100- and pBC12CMV- based plasmids. In order to elucidate the reciprocal nature of Rev/RRE interactions both intra- and inter- strain combinations were essayed. The average results of three independent transfections (mean +/- standard deviation) are illustrated in graphic form in figure 6.8a-c. It is apparent that both the 1514 and EV1 Rev expression vectors significantly increase expression from all three reporter constructs. Thus Rev activity is able to increase expression of the CAT pre-mRNA if an appropriate *cis*-acting responsive element is present. The level of induced activity, however, varies with respect to the viral strain of origin of both the protein and RRE. Thus,

pRev1514 induces significantly higher CAT activity from the cognate pOX100/1514RRE plasmid than does pRevEV1 ($p<0.01$) (6.8a). Although the reverse is also true for induction from pOX100/EV1RRE (6.8b), the difference was found not to be statistically significant ($p>0.2$). Co-transfection with the heterologous pOX/CORRE resulted in higher CAT activity with both MVV strain Rev proteins. Again, although the 1514 protein induced higher enzymatic activity, the difference between the two proteins was not found to be significant. These results are translated into levels of induction of CAT activity (+Rev) over the background (-Rev) for each reporter construct in table 6.1. The highest fold increase in expression is recorded by the 1514 Rev/RRE pair (27.8x background). The 1514 Rev protein is also a better inducer of the conspecific EV1 RRE than of the CO RRE ($p<0.1$). The induction achieved by the EV1 protein is similar with both the MVV RRE constructs, and is less with the CAEV construct.This difference is significant with $p<0.01$ for the 1514RRE and $p<0.1$ for the EV1 RRE: the difference in certainty being a result of the variation observed with the assays with the EV1RRE construct. It should be noted that high backgrounds meant that the fold induction of expression from the pOX100/CORRE plasmid were lower than crude CAT activity would suggest.

Table 6.1: Fold Induction of CAT Expression from pOX100 Vectors by pRev Co-Transfection

pOX100 RRE \ pRev	1514	EV1
1514	27.8 +/- 5.1	10.4 +/- 1.6
EV1	7.6 +/- 1.6	10.9 +/- 4.9
CO	4.8 +/- 1.5	3.9 +/- 1.3

Figure 6.8: Effect of pRev Co-Transfection on CAT Expression from pOX100/RRE Vectors

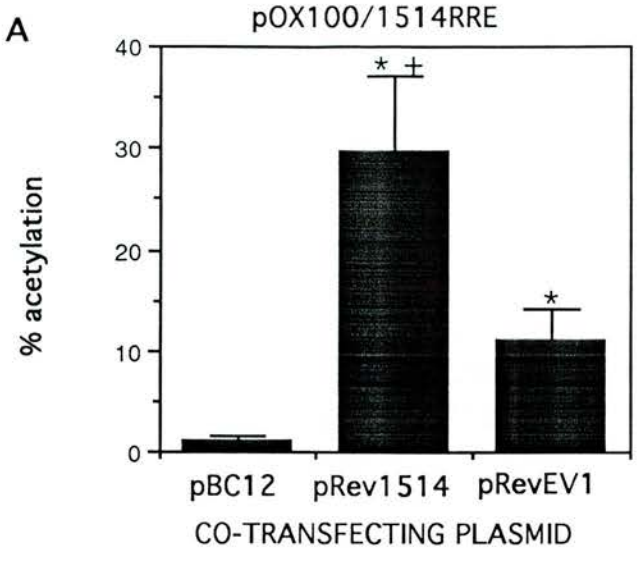
All assays and presented results are as described in figure 6.7a. Significant differences from transfection with pBC12CMV alone are denoted thus: * $p< 0.01$ ** $p< 0.001$. + denotes difference from co-expression with pRevEV1 $p<0.02$.

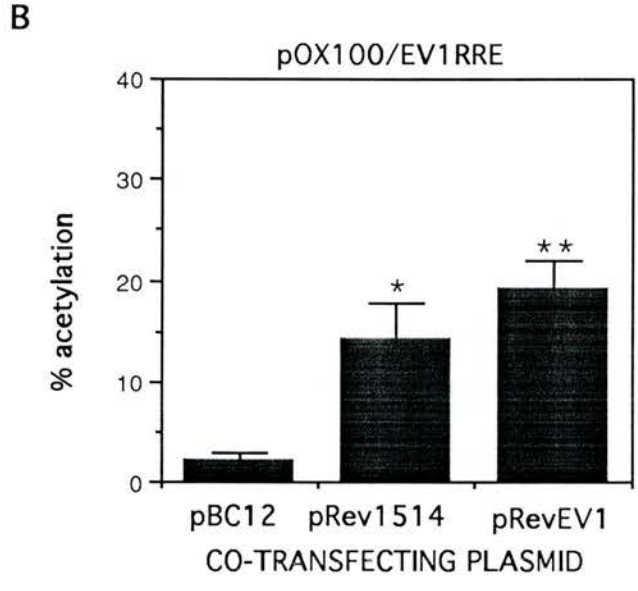
Reporter constructs as follows:

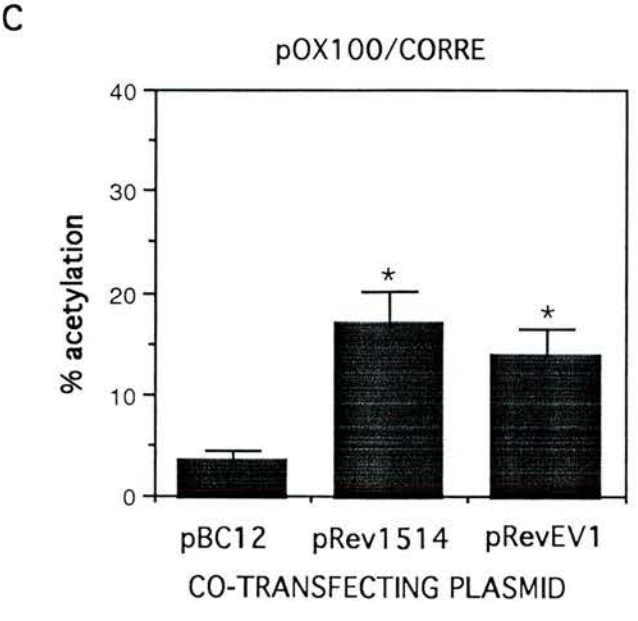
A pOX100/1514RRE

B pOX100/EV1RRE

C pOX100/CORRE


A
pOX100/1514RRE
40
30
20
10
0
% acetylation
* ±
*
pBC12
pRev1514
pRevEV1
CO-TRANSFECTING PLASMID


B
pOX100/EV1RRE
40
30
20
10
0
% acetylation
*
**
pBC12
pRev1514
pRevEV1
CO-TRANSFECTING PLASMID


C
pOX100/CORRE
40
30
20
10
0
% acetylation
*
*
pBC12
pRev1514
pRevEV1
CO-TRANSFECTING PLASMID

6.6 Discussion

The use of eukaryotic transfection techniques to assay for the intracellular activity of the MVV Rev protein has been reported in this chapter. Proteins derived from both the Icelandic 1514 and the British EV1 strain were competent to mediate a significant increase in in CAT activity from reporter constructs. It is assumed that this reflects functional expression of pre-mRNA containing a CAT reporter gene linked to a viral sequence with structural similarities to the characterised HIV RRE. Such activity was not observed in the absence of this element. Although these experiments do not indicate the mechanism of *trans*-activation, by analogy with HIV-1 it may be assumed that MVV Rev acts to correct defects in the expression pathway of pre-mRNA. Confirmation of this hypothesis would be obtained by analysis of the presence and distribution of both unspliced and spliced reporter construct RNA in the presence and absence of Rev co-expression. In conjunction with the *in vitro* binding results discussed in chapter 5, this data is sufficient to define an RNA Rev responsive element located between nucleotides 7935-8244 of EV1. The coincidence of this region with an area of predicted secondary structure strongly supports this conclusion. Insertion of the RRE regions into the assay vector in the antisense orientation could be used to confirm the functional significance of these results. It may also be necessary to repeat these experiments in ovine cells to demonstrate the relevance to naturally occurring situations. Preliminary studies in ovine fibroblasts appeared to indicate poor transfection efficiencies. A similar failure to demonstrate Rev activity by transient transfection in caprine primary cells has been reported (Schoborg *et al.*, 1994); it was suggested that this may be a consequence of poor plasmid replication and stability in these cells.

6.6.1 Reciprocity of the Rev/RRE Interaction

Although both viral proteins tested were able to mediate functional interactions with all three vector constructs containing the putative ungulate lentiviral RREs, the level of up-regulation of expression was found to vary. It was found that both proteins demonstrated greatest activity through the cognate RRE sequence, although further experiments would be required to draw statistically relevant conclusions as to the significance of some of the results. This suggests that the *rev* gene and its responsive element have evolved as a unit, such that

divergence in either sequence is matched by divergence in the other. Given the expected essential nature of the Rev/RRE interaction for viral viability, such co-evolution is not surprising, as the selective pressure to maintain functional interactions will be strong. It might be expected that co-evolving genomic units would vary at a lower rate than other regions, due to such selective pressure. However, the Rev proteins of three sequenced MVV strains, 1514, EV1 and SA-OMVV, demonstrate the greatest degree of dissimilarity of any viral proteins. Examination of the amino acid sequence of the Rev proteins used in this study (figure 6.1e) shows that variation occurs throughout the proteins, and also affects the proposed functional domains. However, it is apparent that amino acid changes within these domains are in most cases of a conservative nature. Thus, there are lysine/arginine substitutions in the basic domain and hydrophobic (valine/methionine/leucine) substitutions in the activation domain. The possible importance of a central hydrophobic core within the basic domain is suggested by the conservative substitution of the 1514 tyrosine residue (position 81) for phenylalanine in EV1. The RREs of 1514 and EV1 are approximately 11.5% divergent at the nucleotide level (figure 6.2b). The overall dissimilarity of the transmembrane glycoprotein (TM) coding region for these viruses is 18.4% (Sargan *et al.*, 1991). It would therefore appear that selection has favoured a relative conservation of the RRE sequence. However, the protein sequence encoded by the RRE region is also more conserved than the overall TM (7.7% against 20.4%) which can be interpreted as the result of evolution to maintain either the RNA or the protein sequence, or both. The CAEV Co strain has an RRE which is equidistant from the MVV sequences, and mediates a similar activation level for both proteins. The unexpected finding that insertion of the CAEV RRE led to an increase in basal CAT activity may be interpreted as interference with repressive sequences present in the surrounding HIV sequence. Interestingly, a report of CAEV Co Rev/RRE function published since the completion of this work has shown that insertion of certain Co sequences into a HIV-1 Gag subgenomic construct also resulted in higher basal activity compared to insertion of the HIV-1 or HTLV-1 response elements (Saltarelli *et al.*, 1994). This finding was not commented on and does not have supporting statistical analysis.

The functional reciprocity of the tested Rev proteins and RREs was therefore complete with respect to presence of function, but incomplete with respect to degree of function. Observed results appeared to correlate with the degree of overall divergence. Thus the close phylogenetic relationship of these viruses is supported by these data. However, it

should be remembered that the complete reciprocity demonstrated by the HTLV-1 Rex protein and the HIV-1 RRE (Rimsky *et al.*, 1988) is the probable result of the fortuitous presence of a high affinity binding site and not a consequence of phylogenetic relatedness. Although the Icelandic neurovirulent K1514 MVV strain (Sonigo *et al.*, 1985) and the CAEV Cork (Co) strain (Narayan *et al.*, 1980; Saltarelli *et al.*, 1990) are recognised as prototypic members of two ruminant lentiviral groups with distinct biological and genetic properties (reviewed in Narayan and Clements, 1989) there is a considerable overlap in the properties of other virus isolates; hence complete classification of ovine/caprine lentiviruses has proven controversial. Thus, partially sequenced isolates from naturally infected sheep in France displayed 4-7% divergence from CAEV Co, but 12-15% divergence from MVV K1514 (Leroux *et al.*, 1995). American ovine lentiviruses cause disease with pulmonary and synovial pathology similar to that of classical CAEV disease (Lairmore *et al.*, 1986). The failure to amplify regions from the other viral strains used in this report is also suggestive of sequence divergence of these viruses. Extension of the Rev functional assays reported here may help elucidate the relationships of these viruses by demonstrating the degree of cross-strain reciprocity.

It has been suggested that gene therapy with *trans*-dominant inhibitory Rev mutants may be a suitable approach to combating lentiviral disease (Malim *et al.*, 1989b). If the mechanism of *trans*-dominant inhibition involves binding competition for high affinity sites, and subsequent lack of multimerisation by mutant molecules (see 1.11.2) then the ability to bind to the RRE of numerous possible strains of infecting virus is essential. If the incomplete reciprocity reported here reflects binding events, which seems possible, then an animal expressing TD Rev mutants of the EV1 strain may not protect against challenge with the 1514 strain. Although, as has been discussed, the degree of ungulate lentiviral divergence is especially high, this may have important implications for human anti-AIDS gene therapy. It should be noted that incomplete reciprocity between HIV strains has not been documented, and that a highly divergent HIV-2 isolate has been shown to be responsive to Rev function of the prototypic HIV-2_{ROD} strain (Kawamura *et al.*, 1992). However, it is also possible that incomplete reciprocity reflects poor multimerisation on heterologous RREs, which may not interfere with gene therapy. Failure to multimerise on the HIV-1 RRE is the mechanism behind the lack of functional activity on this element demonstrated by the SIV/HIV-2 Rev proteins (Garrett and Cullen, 1992; Berchtold *et al.*, 1994). Further research to distinguish these

possibilities is indicated.

The higher activity demonstrated by the 1514 protein may suggest a greater *trans*-activating ability. However, to confirm this studies of the level of expression of each protein in transfected cells would be required. Higher level of expression or greater stability of the 1514 protein may underlie the higher induced activation levels. Experiments designed to identify the effect of titrating the level of co-transfecting pRev plasmid on CAT activity may also be of benefit.

It is impossible from these results to draw any conclusions as to the site of Rev interaction with the RRE. However, progressive truncation of the RRE insert may allow the site of interaction to be pinpointed more exactly. Preliminary results from such experiments might allow the construction of oligonucleotide probes for *in vitro* binding studies.

6.6.2 The pgTAT Assay

The poor signals and resolution of the immunoprecipitated, radiolabelled Tat proteins meant that interpretation of this assay was difficult and contra-indicated its use for analysis of Rev/RRE functional interactions. These difficulties have been encountered by other researchers (R. Fridell, personal communication) using the MVV 1514 Rev/RRE combination. The source of the problem was not identified by attempts to increase resolution. Two possibilities are that *trans*-activation of pre-mRNA expression by MVV Rev in this system is poor, or that technical problems are encountered. The latter possibility would allow possible improvements in this assay. A modified form of pgTAT, pB94, has been constructed: this expresses forms of Tat with greater differences in molecular weight, and may aid discrimination and resolution of the assay (R. Fridell, personal communication). Alternatively, poor transfection efficiencies may have been responsible for the low signal from labelled Tat by reducing the level of expressed protein. Limited proteolytic degradation of the Tat protein may have been responsible for low and indistinct signals. However, it is possible that the fundamental problem with this assay is an inefficient interaction of MVV Rev with cognate RRE in the context of the HIV-1_{HXB3} subgenomic sequence. This may be due to inhibition of the correct formation of the RRE. Replacement of the HIV-1 sequences with a similar subgenomic region of MVV would test this hypothesis, and provide a more appropriate test for MVV Rev function. A molecular clone of the EV1 sequence is not presently available,

however. Discrimination between the possible causes of the poor results demonstrated by this assay may be achieved by analysis of the RNA expression pattern in transfected cells. High level cytoplasmic expression of full length, pre-mRNA in co-transfected cells would indicate that the major problem was of a technical nature. Functional studies of Rev using pgTAT have been scored by RNA analysis (Malim *et al.*, 1988; Lawrence *et al.*, 1991).

6.7 Summary

The *rev* gene and putative responsive element of the MVV EV1 strain were cloned from viral genomic DNA. A RRE was also isolated from a molecular clone of the CAEV Co strain. An attempt to clone these regions from heterologous viral strains proved unsuccessful, a probable consequence of sequence variation. The *rev* gene was cloned into a eukaryotic cell expression vector, and the RREs into the HIV-1 based reporter constructs pgTAT and pOX100/pL. Vectors containing the MVV 1514 strain *rev* gene and RRE were also available. Functional studies of Rev function with the pgTAT assay provided preliminary evidence for 1514 protein interaction with vectors containing the cognate RRE and the EV-1 element. However, poor resolution of results meant that this system was of limited usefulness. In contrast, the CAT assay based on the pOX100 vector proved to be relatively reproducible. Preliminary results indicated that Rev co-expression was sufficient to rescue the expression of the CAT gene if a putative RRE element was present within the CAT intron. A series of reciprocal experiments suggested that although both EV1 and 1514 Rev proteins were able to function through all three tested RREs, the degree of function was greatest with the cognate RRE. Co-evolution of the *rev* gene and Rev responsive element of each strain is suggested by these results. Possible implications for anti-lentiviral gene therapy with *trans*-dominant inhibitory Rev molecules are discussed.

CHAPTER SEVEN

CONCLUSIONS AND FINAL DISCUSSION

7.1 Introduction

The expression and partial functional characterisation of the Rev protein from the British EV1 isolate of the ovine Maedi Visna lentivirus has been reported. Expression of this protein in a yeast system was found to induce a significant degree of cytotoxicity which resulted in a low yield and purity of product. Expression in bacterial cells as a GST fusion gave rise to a reasonable yield and partial product purity. This fusion protein was used in *in vitro* RNA binding studies to delineate a region of the EV1 *env* gene which mediates high affinity binding to GSTRev. Transient transfection of eukaryotic cells with a Rev expression vector and reporter constructs containing the binding element demonstrated that this interaction could mediate the functional expression of genes constitutively expressed at a low level. Reciprocal studies of the functional interaction of Rev proteins and response elements from different viral strains suggested that co-divergence of this axis had occurred.

An extensive discussion has accompanied each chapter throughout this thesis. Thus, this final discussion chapter will concentrate on the possible significance of the observed results, on the relevant literature published since the outset of the research project, and on possible future directions for MVV regulatory gene research.

7.2 Expression of Recombinant EV1 Rev Protein

A source of MVV EV1 Rev protein was required for the attempted functional characterisation of this potentially important regulatory protein. Extraction and purification from virally infected cells was clearly impracticable. Thus, an approach based on the generation of recombinant protein was taken. The choice of which of the multiple expression systems available to use was made on the basis of the biochemical requirements for the expressed protein. Thus, solubility, high purity and maintenance of native conformation were considered important. Systems based on fusion to a carrier molecule were chosen in the hope that the known, beneficial characteristics of the carrier would aid expression and purification (for a review of recombinant expression systems, see Marston, 1986).

The isolated *rev* gene was cloned into expression constructs based on the yeast Ty-VLP and the bacterial pGEX systems. Although correct expression was confirmed in both cases, the yield and purity of product varied considerably. A rapid and devastating toxic effect

was associated with Rev expression in yeast cells. As the Ty-VLP purification protocol involves selection through physical rather than biochemical protein characteristics, it is critically dependent on high protein yield for success. Thus, the purity of p1:Rev protein was low. Attempts to increase yield and purity by altering aspects of the expression and purification protocol met with limited success but did not allow the production of workable protein levels. Expression in the bacterial system was more successful. A moderately high yield and purity of product were achieved. Contaminating molecules were found to correspond to truncated fragments of the full length recombinant protein, and were hypothesised to be the products of proteolysis or mis-translation. By comparison with other reports of the biological activity of GST fusion proteins, the chimaeric protein GSTRev was considered a suitable substrate for functional assays.

7.2.1 Expression of Recombinant Rev/Rex Molecules

At the outset of this project there were few reports of the *in vitro* expression of recombinant lentiviral Rev protein. Subsequently, the production of HIV-1 Rev by a variety of mechanisms and in different cell types has been widely reported. Comparison of these reports with the expression of EV1 Rev achieved in this project may produce suggestions for a future improvement in the purity and yield of recombinant EV1 Rev.

Both direct and fusion-based expression protocols have been utilised. Purification to near homogeneity has been associated primarily with direct expression protocols (Nalin *et al.*, 1990; Wingfield *et al.*, 1991; Zapp *et al.*, 1991). HIV-1 Rev was expressed as a soluble cytoplasmic protein in these systems, and was purified using denaturing techniques, with subsequent refolding. Yields of up to 5% of total cellular protein were achieved (Zapp *et al.*, 1991). Significantly, there are no reports of protein instability using these systems. As direct expression might be more likely to promote instability than fusion to a known stable product, the lack of apparent instability of nonfusion HIV-1 Rev in a soluble form suggests that stability in bacterial cells is an intrinsic property of this protein. Recombinant protein derived from direct expression systems has been shown to retain function, despite the requirement for denaturation. Fusion systems have also been used to produce recombinant Rev. Heaphy and colleagues generated functional protein by fusion to β-galactosidase (1990). This produces inclusion bodies within host cells, which can be isolated by their distinct sedimentation

properties and purified in denaturing conditions. MVV Rev from the 1514 strain has been produced as a β-galactosidase fusion: however the chimaeric protein was purified by excision form a SDS-PAGE gel and used only as an immunogen (Mazarin *et al.*, 1990). Successful fusion to the enzyme β-glucuronidase has also been reported (Daly *et al.*, 1993; Auer *et al.*, 1994). However, the pGEX system has been most widely used as the source of recombinant Rev/Rex protein. HIV-1, HIV-2 Rev and HTLV-1 Rex proteins have been successfully expressed (Bogerd *et al.*, 1991; Malim and Cullen, 1991; Garrett and Cullen, 1992; Hammerschmid *et al.*, 1994; Jensen *et al.*, 1995). However, none of these authors claim purification to homogeneity using the pGEX system. Indeed, in many cases the final purity achieved is not recorded, although Jensen *et al.* (1995) have claimed a figure of 70%. Tiley and Cullen (1992) have published results of expression and binding studies on the Rev protein from the MVV 1514 strain. The fusion protein was reported to be between 25-50% pure, although a SDS-PAGE profile of the protein was not presented. This result is relatively similar to that achieved in this report for the protein of the EV1 strain, and suggests that these proteins have similar characteristics in this system. It is also noticeable that these authors do not report removal of the carrier in either the HIV-1 or MVV fusions by proteolytic cleavage, implying that problems similar to those reported here may have been encountered. It is clear that the problems associated with EV1 Rev expression are by no means unique.

Other findings presented in this study are consistent with reports in the literature. Several authors use BL21 as the host strain for expression (Bogerd *et al.*, 1991; Malim and Cullen, 1991; Hammerschmid *et al.*, 1994). A three-fold difference in HIV-1 Rev yield depending on host strain was reported by Wingfield and colleagues (1991), illustrating the potential importance of the host genotype with respect to encoded proteases (reviewed in Gottesman, 1989). Several groups used synthetic fragments of the *rev/rex* genes, or altered certain codons by site-directed mutagenesis, in order to conform to the parameters set by bacterial codon frequency rates (Nalin *et al.*, 1990; Heaphy *et al.*, 1990; Bogerd *et al.*, 1991).

7.2.2 Alternative Expression Strategies for EV1 Rev

The results obtained here, and by other groups, support the proposition that each protein has unique characteristics, and that estimates as to the outcome of expression studies are not to be relied upon. Properties such as the toxicity of EV1 Rev in *S. cerevisiae*

cells cannot easily be predicted. Direct experimentation is thus an absolute necessity.

Suggestions as to an alternative approach to the synthesis of recombinant Rev may be made based on the experience of other groups and on that gained in this project, although with the above proviso. The functionality of HIV-1 Rev protein derived from denaturing purification systems suggests that these may be useful. In particular, expression of EV1 Rev as an insoluble fusion to a carrier such as β-galactosidase, might be expected to stabilise the protein by the formation of biologically inert inclusion bodies. Substitution of rare bacterial codons in the *rev* sequence for more common synonymous codons may be helpful (see 4.8.3). Elucidation of the mechanism responsible for the presence of truncated molecules in the GSTRev preparations would indicate which of these two strategies would be most beneficial. Purification by inclusion body isolation, protein precipitation, size exclusion chromatography and renaturation by dialysis may be successful. An alternative strategy would be to utilise the system recently described by Orsini and colleagues (1995). HIV-1 Rev was over-expressed in *E. coli* using the bacteriophage λ P_L promoter. Purification was based on the addition of polyethyleneimine, which precipitates nucleic acids and nucleic acid binding proteins, such as Rev. Further purification by chromatography does not require denaturation, which means that the expressed protein retains its authentic structure. Rev was purified to homogeneity by this protocol.

7.2.3 GSTRev as a Reagent for Functional Assays

The decision to use the chimaeric GSTRev protein as a substrate in *in vitro* RNA binding assays was a forced one due to the inability to specifically remove the GST carrier without secondary protease activity. An identical problem was encountered by other authors using a HIV-1 GSTRev fusion (Jensen *et al.*, 1995). However, there are numerous reports of wild type functional activity residing within GST fusions, which would vindicate the approach taken in this project. GST chimaeras have been used as traps to isolate factors which interact with the GST fusion partner. Success has been achieved with fusions to the retinoblastoma gene product and herpes simplex virus type 1 VP16 (Kaelin *et al.*, 1991; Lin and Green, 1991). Essentially normal enzymatic activity is associated with a GST-phosphatase fusion protein (Plutzky *et al.*, 1992). More significantly, several reports on the functionality of distinct RNA binding protein fusions have been published. The polypyrimidine tract binding protein

(hnRNP A) binds correctly to an RNA target within the 5' untranslated region of the hepatitis C virus as a GST fusion (Ali and Siddiqui, 1995). A GST influenza virus NS1 (see 1.18) protein fusion binds with identical characteristics as free protein to the poly(A) containing RNA which is its functional target at low concentrations (Qiu and Krug, 1994). At high concentrations, however, the nonfusion NS1 protein binds target RNA with slightly higher efficiency than the GST-NS1 fusion. There is some reported variation in the relative RNA binding activity of Rev/Rex proteins when expressed as GST fusions. This may reflect differing purification protocols, bacterial host strains or RNA binding assay conditions, and highlights potential problems encountered when comparing data obtained by different research groups. Zapp and co-workers were unable to record significant binding of HIV-1 GSTRev to RRE-RNA, and suggested that the fusion protein was severely defective in functional activity (1991). However, in most cases the differences in activity observed are quantitative rather than qualitative. Steric or conformational restraints imposed by the GST carrier may be responsible for altering the activity of the Rev polypeptide. However, essentially identical RNA binding characteristics using free or GST fusion Rev protein have been reported by other groups (Hammerschmid *et al.*, 1994; Jensen *et al.*, 1995). Minor variations in the primary sequence of the Rev proteins used might result in different higher order structural interactions between the Rev and GST moieties, which in turn could influence Rev function. Alternatively, differences in the presence and concentration of contaminating molecules between preparations might be significant. The fact that reports suggest that only partially pure GSTRev preparations were successfully used in functional studies vindicates the approach adopted in this study, and supports the validity of the reported results.

7.2.4 Toxicity

Expression of p1:Rev within yeast cells was associated with rapid inhibition of culture growth. Preliminary investigation by electron microscopic examination of host cells demonstrated dramatic disruption to the cellular ultrastructure. Although the toxic mechanism and resulting sequence of events are unknown, it is clear that cell death is the endpoint. There was no indication that cytotoxicity was reversible, implying fundamental damage to the cell. Extensive vacuolation within cells may be a cellular defence response to the presence of toxin: the presence of putative recombinant VLPs within vacuoles may support this

hypothesis. However, the irregular vacuolar morphology is perhaps more likely the result of formation by physical upheaval due to the 'imploding' of cellular components. Cross-linking of Rev ligands by the multimeric VLPs could induce such a phenomenon. Alternatively, or in concert, direct disruption of subcellular components by, for example, Rev-mediated VLP blockage of the nuclear pore complex, may be significant.

Although sequestration of Rev binding substrates by recombinant VLPs would certainly be expected to have deleterious consequences for cell viability, the rapidity of toxicity, coupled with the ultrastructural consequences lend support to the hypothetical cross-linking mechanism.The low yield of recombinant VLPs also suggests that ligand sequestration does not sufficiently explain toxicity.

The reduced growth of GSTRev-expressing bacterial cell cultures with respect to uninduced cultures may point to a toxic effect in this system. However, the yield of protein obtained was within the expected range, and the reduction in growth considerably less marked. The possible significance of rare bacterial codons appearing in tandem within the *rev* mRNA have been discussed. In particular, it should be noted that reduced viability of bacterial cells has been associated with the expression of foreign genes possessing consecutive rare codons (Brinkmann *et al.*, 1989). Thus, there is no evidence to link GSTRev to the dramatic toxicity observed in the yeast system, although further study of the consequences for bacterial viability of EV1 *rev* gene expression would be required to draw a firm conclusion. Differences in the consequence of Rev expression must be a product of cell- and/or expression system-specific properties. The functional activity of HIV-1 Rev in yeast cells (Stutz and Rosbash, 1994) implies the presence of appropriate Rev interacting structures and factors. Homologues of the three putative Rev cellular co-factors, eIF-5A, B23 and the putative nucleoporin Rip/Rab (see chapter 1) are not present in prokaryotes. Thus there is an obvious correlation between the presence of Rev ligands and cellular toxicity. However, since monomeric, native HIV-1 Rev does not demonstrate high toxic activity in yeast (Stutz and Rosbash, 1994) the polymeric, particulate form of Ty:Rev VLPs may also be significant.

Rev-mediated cytotoxicity has also been reported to be a feature of overexpression in other cell types, for example during transient transfection of transformed cell lines. Expression of CAEV Rev in COS-1 cells is associated with the appearance of rounded cells exhibiting reduced viability (Schoborg *et al.*, 1994). Nucleolar deformation has been

observed in BHK and COS cells expressing high concentrations of HIV Rev (Nosaka *et al.*, 1993). Subsequent dysfunctional ribosome biogenesis results in nucleolar degradation, and eventual cell death. An intact nucleolar localisation and multimerisation domain are required for this activity; integrity of the activation domain is not essential (Nosaka *et al.*, 1993). Accumulation of B23 within Rev-deformed nucleoli has recently been demonstrated (Miyazaki *et al.*, 1995). It has been speculated that sequestration of B23 by Rev may inhibit the nuclear import activity of the former protein, which in turn would repress ribosome biogenesis. The relevance of this observation to p1:Rev yeast cytotoxicity is uncertain: although it may be a contributory mechanism, inhibition of the yeast B23 homologue would not be expected to result in such rapid structural changes. Nucleolar deformation and cell death were observed in acutely HIV-infected T lymphocytes, but were not observed in chronically HIV-infected monocytic cell lines (Nosaka *et al.*, 1993). Rev protein may be expressed at insufficient levels to promote toxicity in these cells. It is unclear whether Rev plays a significant role in virus-mediated cytotoxicity *in vivo*. Although the recent demonstration of the massive turnover of both virus and host cells in the peripheral circulation of the late stage HIV-infected host strongly suggests immune-mediated cytopathology (Ho *et al.*, 1995; Wei *et al.*, 1995), other mechanisms may be important at other periods. Further investigation into the cytotoxic activity of MVV and HIV Rev proteins may bring valuable insights both into lentiviral pathogenesis and Rev function.

A structural dissection of the domain requirement of MVV Rev for yeast toxicity would presumably give an indication of the mechanisms involved. Injection of synthetic peptides corresponding to the basic domain of HIV-1, -2 and SIV Rev proteins has been shown to induce specific, lethal neurotoxicity in rodents (Mabrouk *et al.*, 1991). This property is not shared by the homologous domain of HTLV-1 Rex, but is characteristic of the basic domains of MVV and HIV Tat proteins (Sabatier *et al.*, 1991; Phillipon *et al.*, 1994). The proposed mechanism of Rev toxicity involves disruption of acidic phospholipids within membrane bilayers, and is associated with the ability of the peptide to form a helical structure in the inorganic phase. This suggests a further possible mechanism for p1:Rev toxicity in yeast; insertion into and disruption of cellular membranes. Such activity might be expected to result in the observed ultrastructural damage occasioned by p1:Rev synthesis. Tentative evidence for the significance of this property in infected individuals has come from the work of Ranki and co-workers, who have identified a correlation between expression of Rev and Nef

proteins in HIV-infected astrocytes and AIDS dementia (1995). Rev toxicity may have a role in the extensive neurological pathology associated with MVV visna (Petursson *et al.*, 1976).

7.3 Rev/RNA Binding

Evidence has been presented for a high affinity, and relatively specific, interaction between EV1 GSTRev and an RNA element with complex predicted secondary structure. The presence of extraneous sequences within the RNA ligand mean that a strict definition of this region as the EV1 Rev response element is not possible. However, the predicted secondary structure is highly conserved amongst three ungulate lentivirus strains, strongly suggestive of functional selective pressure. This structure has been experimentally confirmed as the site of MVV 1514 binding in *in vitro* gel mobility shift assays (Tiley and Cullen, 1992). Binding was observed in the presence of 100ng (approximately 200nM) GSTRev, although a precise comparison with results reported in this thesis is impossible due to the lack of information regarding the purity of the protein preparation, and the absence of extensive protein concentration titration experiments. These authors observed distinct multiple band shifts in the presence of increasing concentrations of GSTRev fusion protein, suggesting that protein multimerisation on the RNA target can occur, as is demonstrated by HIV-1 Rev. Moreover, the binding of GSTRev to labelled specific probe RNA could be competed out with a 400 fold excess unlabelled probe in the presence of excess protein (titration experiments were not performed). RNase protection assays were performed to analyse the Rev/RRE interaction. Protection from cleavage was observed throughout the stemI, stemII and the base of stemIII subregions of the RRE. Comparison of the nucleotides protected by MVV 1514 GSTRev binding with figure 6.3c suggested that these nucleotides were not particularly highly conserved between the three ungulate lentiviral strains.

Binding of the HIV-1 Rev protein to the asymmetric guanine-rich bulge which forms the core binding element may be mediated through a protein helical structure (Tan *et al.*, 1993). Circular dichroism has suggested that the entire binding domain may take helical form (Auer *et al.*, 1994). The carboxy-terminal proximal section of the EV1 and 1514 Rev basic domains is predicted to form helical secondary structure (figure 3.3). Helix destabilising glycine residues may prevent helical formation within the remainder of this domain. Thus, the MVV protein may use only those residues forming helical structure to mediate recognition, or

alternatively, a different structure may be employed. The use of synthetic peptides derived from this region in *in vitro* assays may aid elucidation of the relationship between structure and function. Furthermore, the effect of the observed substitution of arginine and lysine residues between the EV1 and 1514 proteins may be investigated by a similar approach.

Much remains to be learned of the interaction between MVV Rev and its target RNA. In particular, is protein multimerisation required? The work of Tiley and Cullen (1992) would suggest that multimerisation on RNA does occur. Co-operative binding of Rev monomers is essential for functional interaction of HIV-1 Rev with the RRE. Daly *et al.* (1993a) have demonstrated that the RNA binding half life of a Rev molecule is extended three-four fold on binding of a second molecule. Kinetic studies of the MVV Rev/RNA interaction may help understanding of the mechanism of binding specificity, by comparing the on and off rates for specific and non-specific interactions.

7.4 Functional Implications of Rev/RNA Interactions

MVV Rev has been shown to control the production of the Env glycoprotein in infected cells (Tiley *et al.*, 1990). This activity is mediated by the liberation of pre-mRNA encoding the Env protein from nuclear retention, and is thus homologous to the functional mechanism of HIV Rev. Chimaeric proteins containing the MVV basic domain and the HIV-1 activation domain were competent to direct *trans*-activation of MVV structural genes from a subgenomic fragment, provided a MVV RRE structure was present. The reverse situation was also found to be true; thus the leucine-rich domain of MVV Rev was defined as an activation domain which is functionally interchangeable with that of the HIV-1 protein. Mutagenesis of the MVV Rev protein identified functionally important residues within the activation domain; the identity and position of such residues was similar to that seen in HIV-1 (Tiley *et al.*, 1991). However, Tiley *et al.* found that MVV Rev activation domain mutants possessed poor *trans*-dominant inhibitory properties, such that substitution with the activation domain of a HIV-1 TD mutant was required for efficient inhibition of MVV wild type Rev function. This is suggestive of a less modular organisation of the MVV protein, such that compensatory interactions from outside the activation domain could be of relevance. Similar results have been presented for the CAEV Rev protein, which maintains a conserved domain structure to that of MVV Rev (Saltarelli *et al.*, 1994). In contrast to an early study of the subcellular localisation of MVV Rev

protein (Mazarin *et al.*, 1990), a conventional nuclear/nucleolar distribution of the Rev proteins of both MVV and CAEV has been reported (Tiley *et al.*, 1990; Schoborg and Clements, 1994; Schoborg *et al.*, 1994). The essential requirement of a functional *rev* gene for both MVV and CAEV viral viability has recently been established (Schoborg and Clements, 1994; Toohey and Haase, 1994). Thus, the overlap in the biological properties of the ungulate lentiviral Rev proteins and the HIV protein has so far shown to be almost complete, with the possible exception of the genesis of inhibitory activation domain mutants.

7.5 Reciprocal Rev/RRE Interactions

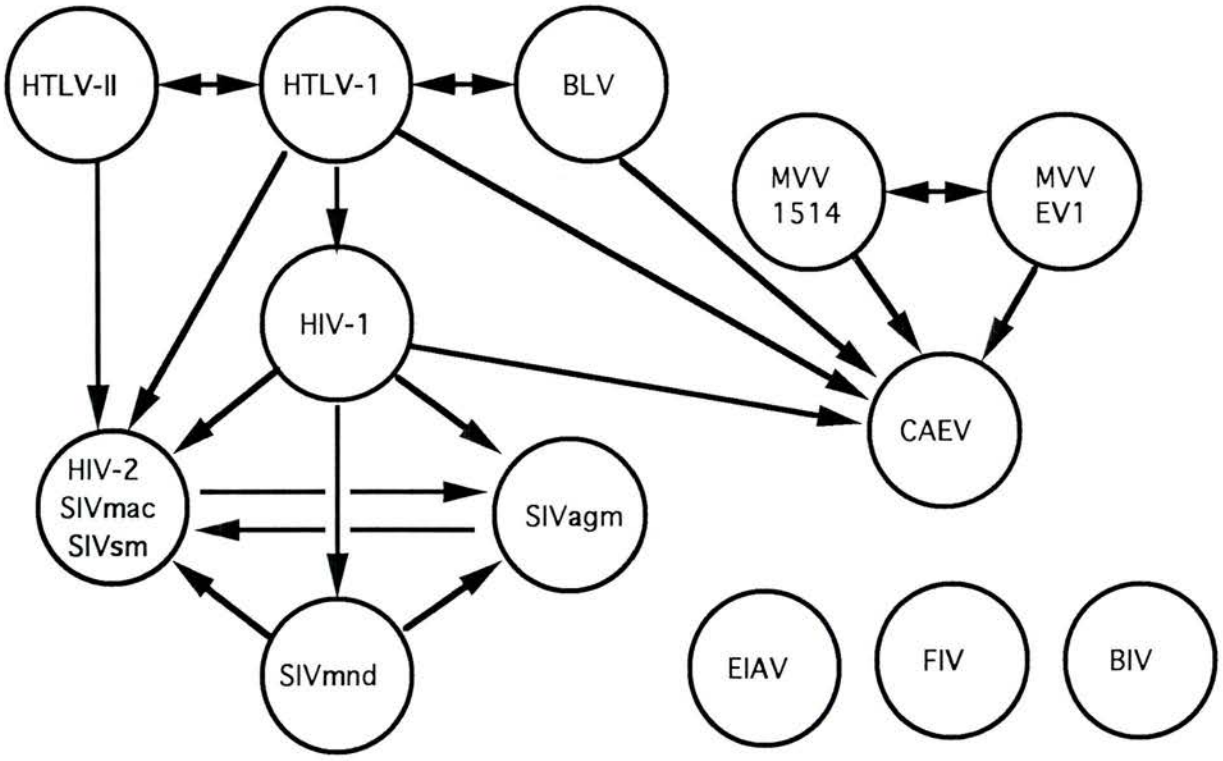
The two strains of MVV used in this thesis, EV1 and 1514, were found to be competent for function through the heterologous RRE. Similarly, both proteins were able to *trans*-activate a gene linked to the CAEV RRE. However, the degree of activity was highest with the cognate RRE. Thus, there appears to have been co-divergence of the Rev/RRE elements from each of these viruses. Heterologous interactions between retrovirus Rev/RREs are illustrated by figure 7.1. The HTLV-1 Rex and HIV-1 Rev proteins are functionally promiscuous, able to mediate activity through the response elements of a range of other viruses. The more fastidious requirements of these other viruses may be at the level of RNA binding, multimerisation or co-factor interactions. The CAEV RRE seems particularly susceptible to the Rev proteins of other viral species (Saltarelli *et al.*, 1994): this does not extend to the MVV RRE (Tiley *et al.*, 1991). Elucidation of the mechanism behind this phenomenon may aid the study of the biochemistry of the Rev/RRE interaction.

7.6 Future Prospects

Much remains to be learned about the molecular biology of the MVV Rev/RNA interaction, and of the functional mechanisms which Rev utilises to exert regulatory control over viral replication. However, much of this work, though important, may involve recapitulation of the experimental approaches used thus far to analyse the HIV equivalents. To date, the observed conservation of the biology of MVV/CAEV and HIV Rev regulatory systems has been remarkable for such sequence-divergent viruses. It may be suggested that this functional homology will continue to be observed. Thus, it may be appropriate to concentrate

Figure 7.1: Compatibility of Rev/RRE Axis of Diverse Complex Retroviruses

Figure illustrates the experimentally defined interactions between the Rev/Rex proteins of complex retroviruses and the respective response elements. Arrow indicates that the Rev proteins of the originating virus is competent to mediate function through the response element of the indicated virus. For example, HIV-1 Rev can function through the HIV-2 RRE; this function is not reciprocal. Full reciprocity is indicated by a double headed arrow. For simplicity, no indication of the degree of function is indicated in this diagram. The EIAV, FIV and BIV proteins have yet to be comprehensively studied, although EIAV and HIV-1 Rev/RRE systems are incompatible (Fridell *et al.*, 1993). Information derived from this thesis, Rimsky *et al.*, 1988; Felber *et al.*, 1989b; Garrett and Cullen, 1992; Sakai *et al.*, 1993; Saltarelli *et al.*, 1994.


HTLV-II
HTLV-1
BLV
MVV
1514
MVV
EV1
HIV-1
CAEV
HIV-2
SIVmac
SIVsm
SIVagm
SIVmnd
EIAV
FIV
BIV

on topics for which the distinct nature of MVV may be of benefit in the overall study of Rev.

It has been proposed that transgenic sheep expressing a mutant form of the MVV Rev protein may be protected from viral infection (Tiley *et al.*,. 1991). Such an experiment would be of interest for the potential development of gene therapeutical approaches against human AIDS. The presence, degree and inter-strain nature of protection would be important variables to consider. Moreover, it would be of interest to identify potential cytotoxic activity in wild-type and mutant transgenic animals. It is possible that over-expression of a *rev* transgene could prove cytotoxic, resulting in a failure to obtain transgenic animals. Sheep expressing a MVV *env* transgene have been reported (Clements *et al.*, 1994).

To date, much of the research on Rev function has been centred on *in vitro* or cell culture studies which may not provide fully comprehensive data as to the importance of Rev for viral replication, persistence and pathogenesis. Experimental infection of sheep with biological clones of MVV could be developed to identify the presence and functional significance of *rev* variation during infection. It would be of interest to determine whether inactivating *rev* mutations occur, as has been reported for HIV-1 infection (Iversen *et al.*, 1995).

Finally, the identification of co-factors for Rev function might allow the effect of ablation of such factors in an infected host to be studied. The human nucleoporin-like molecule rip/rab (1.15.2) which acts as a Rev co-factor in the nucleocytoplasmic export of viral RNA, has been shown to bind the MVV activation domain (Bogerd *et al.*, 1995). Isolation of an ovine homologue of this protein, and analysis of the requirement of the protein for cell viability may result in the development of transgenic 'knock-out' animals failing to express this factor. The susceptibility of such animals to MVV challenge would be of considerable interest in the development of human gene therapeutics.

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